LUMINANCE FLICKER-INDUCED RETINAL VASODILATION IN HUMANS WITH AND WITHOUT TYPE 1 DIABETES

Jonathan Edward Noonan BEc BSc(Hons) MBBS

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Department of Ophthalmology
Faculty of Medicine, Dentistry and Health Science
The University of Melbourne

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ABSTRACT

PURPOSE
Luminance flicker-induced retinal vasodilation is reduced in people with type 1 diabetes compared with non-diabetic controls. However, the mechanisms of this response and causes for its reduction are not well understood. I therefore investigated potential acute causes of reduced luminance flicker-induced retinal vasodilation in people with and without type 1 diabetes. My doctoral project had four specific aims: 1) To investigate the effect of repeated testing on luminance flicker-induced retinal vasodilation in healthy people; 2) To study the effect of ambient lighting on luminance flicker-induced retinal vasodilation in healthy people; 3) To determine the importance of epoxyeicosatrienoic acids (EETs) and prostaglandins (PGs) in luminance flicker-induced retinal vasodilation in healthy people; and 4) To study the effect of glucose levels and antioxidant treatment on retinal flicker responses, ganglion cell function and systemic arterial elasticity in people with type 1 diabetes.

METHODS
My doctoral project involved a total of 72 participants (60 non-diabetic and 12 with type 1 diabetes). I first assessed the impact of repeated testing with five or 30 minutes between tests on luminance flicker-induced retinal vasodilation in 20 healthy people. Next, I used a balanced crossover study investigated the effect of reduced and normal ambient lighting on these responses in another 20 healthy people. In my third study, I used oral fluconazole 400 mg and dispersible aspirin 600 mg to investigate the role of EETs and PGs, respectively, in healthy luminance flicker-induced retinal vasodilation. Twelve healthy people participated in a balanced crossover study with three treatments (no drug, fluconazole and aspirin). In addition, six healthy people, each for fluconazole and aspirin, were followed at 30-minute intervals for two hours after drug ingestions. My final study involved 12 otherwise healthy people with type 1 diabetes. I investigated luminance flicker-induced retinal vasodilation, retinal ganglion cell function and systemic arterial elasticity at baseline, euglycaemia (plasma glucose ≈ 6 mmol/l) and hyperglycaemia (plasma glucose ≈ 15 mmol/l). Participants were seen twice: once with vitamin C (2 g intravenous) and once with placebo immediately prior to the initiation of hyperglycaemia. Retinal vasodilation in response to 20 seconds of 12.5 Hz luminance flicker with green light was assessed with the Dynamic Vessel Analyzer (DVA) after
pupil dilation with tropicamide 1%. Ganglion cell activity was measured from the pattern electroretinogram (PERG) transient (1 Hz stimulus) and steady-state (8.33 Hz) responses to black and white check reversals without pupil dilation. Systemic arterial elasticity was also assessed by radial artery pulsewave tonometry with the HD/Pulsewave CR-2000. Participant characteristics were compared by analysis of variance (ANOVA) for continuous variables or Fisher’s exact test for categorical variables. Within-subject changes in group means were assessed by ANOVA. Significant differences identified by ANOVA were explored by post-hoc Student’s *t*-tests with corrections for multiple comparisons where necessary. Within-subjects Pearson’s correlation coefficients were assessed in my final study for luminance flicker-induced retinal vasodilation, ganglion cell function and systemic arterial elasticity. Two-tailed *P* < 0.05 was considered statistically significant. All statistical analyses were performed in STATA version 12.1.

**RESULTS**

**Aim 1**

Twenty healthy people aged (mean [standard deviation [SD]]) 33.1 (5.7) years were studied. Maximum luminance flicker-induced arteriolar dilations were 3.2 (2.1) % initially, 2.4 (1.6) % after five minutes of rest and 3.4 (2.1) % after 30 minutes of rest (*P* < 0.001 by ANOVA). Compared with the first test, arteriolar dilations were reduced after five minutes (*P* = 0.02 after Bonferroni’s adjustment), but not after 30 minutes of rest (*P* > 0.05). Maximum venular dilations were 4.3 (1.3) % initially, 3.8 (1.6) % after five minutes of rest and 4.4 (1.7) % after 30 minutes of rest (*P* > 0.05 by ANOVA). Pre-flicker vessel diameters were unchanged between tests (*P* > 0.05 for arterioles and venules).

**Aim 2**

Twenty healthy people aged 32.8 (6.5) years were studied. Maximum luminance flicker-induced retinal arteriolar dilations were 4.8 (2.3) % and 4.1 (1.9) % under reduced and normal ambient lighting, respectively (*P* = 0.02 by ANOVA). Corresponding maximum venular dilations were 5.4 (1.8) % and 5.1 (2.1) % under reduced and normal ambient lighting, respectively (*P* > 0.05). Pre-flicker arteriole and venules diameters were unchanged between tests (*P* > 0.05 for both).

**Aim 3**

Twenty healthy people aged 25.6 (4.6) were studied. In 12 crossover study participants, maximum luminance flicker-induced retinal arteriolar and venular dilations without
drug administration were 4.4 (2.0) % and 4.6 (1.7) %, respectively. Neither fluconazole nor aspirin affected these responses ($P > 0.05$ for both by ANOVA). Pre-flicker arteriole and venule diameters without drug administration were 120 (11) measurement units (MU) and 146 (17) MU, respectively. Fluconazole reduced pre-flicker venule diameters by (mean ± 95% confidence interval [CI]) 5 ± 4 MU ($P = 0.02$ after Dunnett’s adjustment). In six participants, fluconazole did not affect arteriolar or venular dilations or pre-flicker arteriole diameters ($P > 0.05$ for all) but did reduce pre-flicker venule diameters over two hours ($P < 0.001$ by ANOVA). Aspirin did not affect arteriolar or venular dilations or pre-flicker diameters in six participants over two hours ($P > 0.05$ for all).

_Aim 4_

Twelve otherwise healthy people with type 1 diabetes aged (median [interquartile range [IQR]]) 24 (20.5 – 30) years with 10.5 (3.8 – 17) years of diabetes were studied. Plasma glucose levels decreased from (mean [SD]) 8.4 (3.6) mmol/l at baseline to 6.1 (0.8) mmol/l at euglycaemia ($P < 0.01$). Plasma glucose levels then increased during hyperglycaemia to 15.0 (1.9) mmol/l with vitamin C and 14.4 (0.8) mmol/l with placebo (both $P < 0.001$ vs. euglycaemia). Insulin levels increased from 65 (24) pmol/l at baseline to 334 (63) pmol/l at euglycaemia ($P < 0.001$) and were stable thereafter. Euglycaemic clamp increased retinal venule maximum dilation by 1.7 (2.0) % and area under the curve (AUC) during flicker by 28.7 (28.9) % x sec. compared to baseline (both $P < 0.01$). No change in flicker responses was observed between euglycaemia and either hyperglycaemia arm or between vitamin C and placebo hyperglycaemia arms. Ganglion cell function and systemic arterial elasticity were unaffected across glucose conditions ($P > 0.05$ for all). The one exception was the ratio of the second harmonic frequency (2F) amplitude with 0.8° check sizes to the amplitude with 7° check sizes from the PERG steady-state response. The ratio increased from 1.1 (0.3) at baseline to 1.4 (0.6) after hyperglycaemia with vitamin C ($P < 0.05$). In within-subjects correlation analyses, the arteriole AUC during flicker was positively correlated with plasma glucose levels ($r = 0.27$, $P = 0.04$), although this correlation became non-significant when data from hyperglycaemia with vitamin C were excluded. Arteriole and venule maximum dilations and AUC during flicker were positively correlated with free insulin levels, although only venule dilation responses remained significant after data from hyperglycaemia with vitamin C were excluded. Large artery elasticity (LAE) was positively correlated with arteriole maximum dilations ($r = 0.26$, $P = 0.04$) and AUC
during flicker ($r = 0.26$, $P = 0.04$) and these correlations remained significant after the exclusion of data obtained after vitamin C treatment. Both LAE ($r = -0.32$, $P = 0.01$) and small artery elasticity (SAE) ($r = -0.35$, $P = 0.01$) were negatively correlated with systolic blood pressure. These correlations were unchanged by the exclusion of vitamin C data. In PERG tests, the N95 amplitude was positively correlated with diastolic blood pressure, the $0.8^\circ$ 2F amplitude was negatively correlated with systolic blood pressure and the $7^\circ$ 2F amplitude was negatively correlated with insulin levels. After the exclusion of vitamin C data, the correlation between the $0.8^\circ$ 2F amplitude and systolic blood pressure was not statistically significant.

**CONCLUSIONS**

Several factors may impact on luminance flicker-induced retinal vasodilation in humans. Arteriolar responses, in particular, are reduced with repeated stimulation and higher ambient lighting. This may reflect light adaptation in photoreceptors and lower modulation depths during luminance flicker, respectively, with secondary reductions in ganglion cell activation. My data do not support a major role for EETs and PGs in luminance flicker-induced retinal vasodilation. Nitric oxide remains the most likely signalling molecule and dysfunction in its signalling pathways may contribute to reduced responses in type 1 diabetes. Increased venule dilations during euglycaemic clamp suggest that glucose normalisation with insulin improves retinal microvascular function. Furthermore, antioxidants such as vitamin C may improve retinal neurovascular function in adults with type 1 diabetes. This finding indirectly implicates oxidative stress as a contributor to impaired retinal neurovascular function in type 1 diabetes. My doctoral project highlights several important factors that may reduce luminance flicker-induced retinal vasodilation and may lead to its use as a clinical marker of neurovascular function in retinal diseases.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2F</td>
<td>Second harmonic frequency</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end-products</td>
</tr>
<tr>
<td>AMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARVO</td>
<td>Association for Research in Vision and Ophthalmology</td>
</tr>
<tr>
<td>AREDS</td>
<td>Age-Related Eye Disease Study</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCVA</td>
<td>Best-corrected visual acuity</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CERA</td>
<td>Centre for Eye Research Australia</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DME</td>
<td>Diabetic macular oedema (abbreviated for USA spelling)</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>DVA</td>
<td>Dynamic Vessel Analyzer</td>
</tr>
<tr>
<td>EDIC</td>
<td>Epidemiology of Diabetes Interventions and Complications</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EETs</td>
<td>Epoxyeicosatrienoic acids</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ERG</td>
<td>Electroretinogram</td>
</tr>
<tr>
<td>ETDRS</td>
<td>Early Treatment Diabetic Retinopathy Study</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectroscopy</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Haemoglobin A1c</td>
</tr>
<tr>
<td>HREC</td>
<td>Human Research and Ethics Committee</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICCs</td>
<td>Intra-class correlation coefficients</td>
</tr>
<tr>
<td>IOP</td>
<td>Intraocular pressure</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>ISCEV</td>
<td>International Society for Clinical Electrophysiology of Vision</td>
</tr>
<tr>
<td>JDRF</td>
<td>Juvenile Diabetes Research Foundation</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N(^G)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>N(^G)-monomethyl-L-arginine</td>
</tr>
<tr>
<td>LAE</td>
<td>Large artery elasticity</td>
</tr>
<tr>
<td>LGN</td>
<td>Lateral geniculate nucleus</td>
</tr>
<tr>
<td>LogMAR</td>
<td>Logarithm of the minimum angle of resolution</td>
</tr>
<tr>
<td>mfERG</td>
<td>Multifocal electroretinogram</td>
</tr>
<tr>
<td>NAD(^3)</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>OPs</td>
<td>Oscillatory potentials</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>PERG</td>
<td>Pattern electroretinogram</td>
</tr>
<tr>
<td>PGs</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>PhNR</td>
<td>Photopic negative response</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RBX</td>
<td>Ruboxistaurin</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>RVEEH</td>
<td>Royal Victorian Eye and Ear Hospital</td>
</tr>
<tr>
<td>SAE</td>
<td>Small artery elasticity</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
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</table>
UDP-GlcNAc Uridine diphosphate-N-acetylglucosamine
UKPDS United Kingdom Prospective Diabetes Study
VEGF Vascular endothelial growth factor
VEP Visual evoked potential
DECLARATION

This is to certify that:

• the thesis comprises only my original work towards the PhD except where indicated in the Preface,
• due acknowledgement has been made in the text to all other material used,
• the thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

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Dr Jonathan E Noonan BEc BSc(Hons) MBBS
PREFACE

The material presented in this doctoral thesis represents my own original work. The exceptions to this statement are: 1) minor parts of my original research papers that have been accepted in peer-reviewed journals and included in lieu of traditional thesis chapters; and 2) minor parts of my original research paper, under review at the time of writing, presented in chapter 7. Descriptions of the contributions to each part of my thesis are included below.

* Indicates contribution of the candidate.

CHAPTER 4
Status: Published
Full title: Retinal arteriolar dilation to flicker light is reduced on short-term re-testing
Journal: Investigative Ophthalmology and Visual Science
Volume/pages: 54/7764-7768
Contributions: Noonan JE* (66%). Reviewed the literature, designed the study, obtained ethics approvals, recruited participants, performed the experiments, collected and analysed the data, prepared and reviewed the manuscript.
Lamoureux EL (14%). Assisted with the study design, reviewed the manuscript.
Nguyen TT (8%). Provided equipment training, reviewed the manuscript.
Man REK (5%). Reviewed the manuscript.
Best WJ (2%). Reviewed the manuscript.
Wang JJ (5%). Reviewed the manuscript.

CHAPTER 5
Status: Published
Full title: Flicker-induced retinal arteriole dilation is reduced by ambient lighting
Journal: Investigative Ophthalmology and Visual Science
Volume/pages: 55/5476-5481
Contributions: Noonan JE* (66%). Reviewed the literature, designed the study, obtained ethics approvals, recruited participants, performed the experiments, collected and analysed the data, prepared and reviewed the manuscript.
Lamoureux EL (14%). Assisted with the study design, reviewed the manuscript.
Dusting GJ (8%). Assisted with data analysis. Reviewed the manuscript.
Nguyen TT (5%). Reviewed the manuscript.
Man REK (5%). Reviewed the manuscript.
Best WJ (2%). Reviewed the manuscript.

CHAPTER 6

Status: Published
Full title: Flicker light-induced retinal vasodilation is unaffected by inhibition of epoxyeicosatrienoic acids and prostaglandins in humans
Journal: Investigative Ophthalmology and Visual Science
Volume/pages: 55/7007-7013
Contributions: Noonan JE* (66%). Reviewed the literature, designed the study, obtained ethics approvals, recruited participants, performed all imaging, collected and processed blood samples until ready for transport, analysed the data, prepared and reviewed the manuscript.
Lamoureux EL (10%). Assisted with the study design, reviewed the manuscript.
Dusting GJ (4%). Assisted with the study design, reviewed the manuscript.
Nguyen TT (3%). Reviewed the manuscript.
Jenkins AJ (3%). Assisted with the study design, reviewed the manuscript.
Man REK (2%). Reviewed the manuscript.
Best WJ (1%). Reviewed the manuscript.
Dias DA (2%). Quantified fluconazole and salicylic acid levels in plasma.
Jayasinghe NS (2%). Quantified fluconazole and salicylic acid levels in plasma.
Roessner U (2%). Quantified fluconazole and salicylic acid levels in plasma.
Januszewski A (4%). Performed prostaglandin $E_2$ metabolite assays.
Xie J (1%). Gave statistical advice.

CHAPTER 7
Status: Submitted
Full title: Retinal neuronal and vascular function in type 1 diabetes during euglycaemic and hyperglycaemic clamp
Journal: Investigative Ophthalmology and Visual Science
Volume/pages: N/A
Contributions: Noonan JE* (66%). Reviewed the literature, designed the study, obtained ethics approvals, recruited participants, performed functional tests, obtained retinal photographs, performed eye tests (except refraction), cannulated participants, collected blood samples, collected and analysed data, prepared and reviewed the manuscript.
Lamoureux EL (6%). Assisted with the study design, reviewed the manuscript.
Ward GM (8%). Assisted with the study design, supervised glucose clamps, reviewed the manuscript.
Sarossy M (6%). Designed PERG protocols, assisted with data analyses, reviewed the manuscript.
Man REK (2%). Reviewed the manuscript.
Nguyen TT (2%). Reviewed the manuscript.
Jenkins AJ (2%). Reviewed the manuscript.
Dusting GJ (2%). Reviewed the manuscript.
Gooley JL (5%). Processed blood samples, performed glucose and insulin assays.
Orthoptists (various; 1%). Refracted participants if required.
I wish to thank the National Health and Medical Research Council (NHMRC) for my Medical Postgraduate Scholarship ID1038701. Their generous support over the duration of my PhD provided funding for my personal living expenses, research-related travel and consumables and the printing of my thesis. Most importantly, my scholarship allowed me to focus on my research by reducing my need to engage in additional paid work.

I received two travel grants during my candidature to help further my professional development. I wish to thank the Association for Research in Vision and Ophthalmology (ARVO) for an International Travel Grant to present my poster entitled “Retinal arteriolar dilation to flicker light is reduced with repeated stimulation” at the 2013 Annual Meeting in Seattle, USA. Their support allowed me to experience my first conference presentation and learn of the latest developments in ophthalmology research.

In my final year of candidature, I received a Melbourne Abroad Travelling Scholarship from the University of Melbourne. This award funded my travel to the ARVO 2014 Annual Meeting in Orlando, USA, to present my poster entitled “Flicker light-induced retinal vasodilation is unaffected by inhibition of epoxyeicosatrienoic acids and prostaglandins in humans” and to the Advanced Retinal Imaging Alliance in Rochester, USA, to visit their institute and give a presentation about my doctoral research.

I received two significant project grants that allowed me to pursue my second and third aims. I wish to thank the Royal Victorian Eye and Ear Hospital (RVEEH) Small Grants Committee for their generous support of my project entitled “Mechanisms of retinal functional hyperaemia in humans”. This grant provided funding for essential medical supplies, laboratory tests and participant travel allowances, without which the feasibility of my project would have been severely compromised. I must also thank the Juvenile Diabetes Research Foundation (JDRF) Australia for their Type 1 Diabetes Clinical Research Network Pilot and Feasibility Grant. Their generous support made possible my project entitled “Retinal vascular function during hyperglycaemia and the role of vitamin C” by providing funding for the personnel, consumables and equipment necessary to safely carry out my hyperglycaemic clamp experiments.

I wish to thank the Centre for Eye Research Australia (CERA) for supporting my research and awarding me the Award for an Outstanding Contribution of a Student at the CERA Scientific Exchange in 2014. I was humbled to be recognised alongside
CERA’s talented researchers and extremely grateful for this acknowledgment of my doctoral work.

I must pay a great deal of thanks for the support and encouragement provided by my primary supervisor, A/Prof Ecosse L Lamoureux, and my co-supervisors, Prof Jie Jin Wang, Prof Alicia J Jenkins, Prof Gregory J Dusting and A/Prof Glenn M Ward, over the course of my doctoral project. Their supervision was instrumental to the development of my research skills and provided a supportive environment where I could design and test my own research ideas.

Finally, I want to thank my partner, Cathrine, for keeping me sane throughout the early mornings and long days as I completed my experiments and chipped away at this rather large document. Rest assured, the three years of my PhD candidature is not something I will be repeating again in the foreseeable future.
PUBLICATIONS AND PRESENTATIONS

1.1.1 Publications related to the thesis

Published/in press/accepted


Under revision/under review


1.1.2 Other publications by the candidate


### 1.1.3 Published abstracts


1.1.4 Presentations
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CHAPTER 1. INTRODUCTION
1.1 Introduction

Retinal arterioles and venules dilate when stimulated with flickering light, a phenomenon described as ‘functional hyperaemia’ or ‘neurovascular coupling’\textsuperscript{1-3}. Vasodilation occurs within seconds of flicker light stimulation and quickly reverses upon its cessation. Transient constriction is observed in arterioles, but not venules, for several seconds after withdrawal of the stimulus\textsuperscript{1}. Hyperaemia during flicker stimulation is thought to be a compensatory mechanism for the increased metabolic demands of activated retinal neurons, particularly retinal ganglion cells.

Retinal haemodynamic reactions to flickering light are highly dependent on the stimulus characteristics. Generally, flicker stimuli can be grouped as either luminance or chromatic flicker. Luminance flicker describes stimuli whose luminance is modulated while holding wavelengths constant; chromatic flicker describes stimuli whose wavelength is modulated while holding luminance constant. Flicker stimuli may include both luminance and chromatic components, although this makes it harder to selectively stimulate visual pathways of interest. Additional determinants of the retinal haemodynamic response to flicker light include the contrast between light and dark phases, wavelengths of light, frequency of flicker and even the light adaptation state of the retina\textsuperscript{1}. Consideration of how different types of ganglion cells respond to particular flicker parameters is important for linking flicker stimuli with functional responses.

Previous work has argued that flicker light-induced retinal vasodilation may reflect endothelial function\textsuperscript{4}. This argument is based on observations that nitric oxide is an important mediator of hyperaemic responses in the optic nerve and retina\textsuperscript{5-7}. However, nitric oxide production is not unique to endothelial cells. Nitric oxide is produced by two calcium-dependent enzymes: neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). eNOS, the enzyme found in endothelial cells, is roughly half as prevalent as nNOS in the retina\textsuperscript{8}. Experimental studies have isolated nNOS in inner retinal neurons, including bipolar, amacrine and ganglion cells\textsuperscript{9}. Observations that nitric oxide is released in the inner plexiform layer during light stimulation and produced by retinal ganglion cells with glutamate, the retina’s main excitatory neurotransmitter, suggest that ganglion cells may be primary mediators of flicker light-induced vasodilations\textsuperscript{8, 10}. This hypothesis is further supported by increased ganglion cell activity in response to luminance flicker\textsuperscript{11}. Signalling molecules other than nitric oxide, such as arachidonic acid metabolites
from retinal glial cells, may also contribute to luminance flicker-induced retinal vasodilations\(^2\). \(^3\). The mechanisms of this phenomenon are therefore far from completely understood.

Clinical interest in the retinal vascular response to luminance flicker stimulation has been generated from several cross-sectional studies that showed reduced vasodilation in people with diabetes mellitus ("diabetes") compared with non-diabetic controls\(^{12-14}\). The chronic hyperglycaemia of diabetes causes progressive damage over years to various ocular structures. Diabetic eye damage may manifest clinically in several ways including diabetic retinopathy (DR) or the accelerated progression of cataract or glaucoma\(^{15}\). Of these ocular complications, DR is the most widely recognised and is the most common cause of blindness in adults aged 20-74 years in the United States\(^{16}\). In people with diabetes, reduced retinal vasodilations during flicker stimulation were independently associated with DR\(^{13}\). Moreover, reduced vasodilations have been observed in people with diabetes prior to the evolution of detectable DR lesions\(^{17-19}\). Flicker light-induced retinal vasodilation may therefore represent a sensitive marker of dynamic retinal function in people with diabetes and provide a useful indication of retinal damage when combined with traditional quantitative measures of DR severity.

The Diabetes Control and Complications Trial (DCCT)\(^{20}\) and the United Kingdom Prospective Diabetes Study (UKPDS)\(^{21}\) using patients with type 1 and type 2 diabetes, respectively, clearly established a strong link between glucose control and the risk of diabetes complications such as DR. Together with diabetes duration, long-term glucose control is now recognised by ophthalmologists as the major risk factor for DR\(^{22,23}\). High intracellular glucose levels are thought to provoke the generation of mitochondrial oxidative stress, which then activates several damaging biochemical pathways\(^24\). Indeed, evidence of hepatic oxidative stress can be found after only a few hours of hyperglycaemia in rats\(^{25}\). In humans, haemodynamic impairment during hyperglycaemia appears to involve reduced bioavailability of nitric oxide\(^{26}\). If nitric oxide is an important mediator of flicker light-induced retinal vasodilation, as suggested by animal and human studies\(^5\)\(^-\)\(^7\), smaller responses in people with diabetes could reflect impaired nitric oxide bioavailability induced by oxidative stress. If true, higher levels of oxidative stress in people with diabetes may represent a promising therapeutic target. The Age-Related Eye Disease Study (AREDS) found that treatment with antioxidants and vitamins could delay the progression of some forms
of age-related macular degeneration (AMD)\textsuperscript{27}. Whether antioxidants are of similar benefit in DR from type 1 diabetes is an important question to address.

It could be hypothesised that reduced retinal vasodilation during flicker stimulation in people with diabetes is related to the long-term disease pathogenesis. However, our knowledge of the specific mediators of this response is limited, and even less is known about the causes for its impairment. This lack of understanding is a major barrier to the routine longitudinal assessment of flicker light-induced retinal vasodilation in people with diabetes. With the advancement in modern technology and imaging, several novel areas of research could be proposed to elucidate the mechanisms underpinning functional hyperaemia.

First, \textit{in vivo} imaging of the retinal flicker response is possible using the DVA system (IMEDOS, Jena, Germany), which allows accurate measurement of retinal vessel diameters in real time through a dilated pupil\textsuperscript{28, 29}. Short-term reproducibility of the flicker response was assessed in two studies with 30 to 60 minutes between consecutive tests\textsuperscript{30, 31}. Substantial correlations were found between arteriolar responses ($R^2 = 0.85 – 0.94$), whereas venular responses were more variable ($R^2 = 0.19 – 0.80$). Neither study evaluated the intra-class correlation coefficients (ICCs) for arteriole or venule dilations, which is a better marker of reproducibility than correlations. Furthermore, no study has considered the short-term impact of repeated stimulation on vessel responses. This information is important for researchers wishing to investigate the effects of pharmacological interventions in repeated measures studies or where difficult data collection may necessitate several measurement attempts. Therefore, \textbf{Aim 1 of my research project assessed the impact on flicker light-induced retinal vasodilation of repeat testing after very short rest periods.}

Tests of flicker light-induced retinal vasodilation are ideally conducted in a darkened room\textsuperscript{13, 14}. However, this is not always feasible. I found that in the course of my doctoral project, for patient safety reasons, I would likely need to perform some tests of the retinal flicker response under normal ambient lighting conditions. In the second study of my doctoral work, I \textbf{therefore investigated in Aim 2 whether normal or darkened levels of room lighting affect the magnitude of luminance flicker-induced retinal vasodilation.}

Nitric oxide has been implicated as the principal mediator of retinal vasodilation during flicker stimulation. Animal and human studies have found that nitric oxide is released in the retina during flicker stimulation and inhibition of nitric
oxide synthase (NOS) reduces vessel dilations\textsuperscript{5-7}. However, recent laboratory work suggests that arachidonic acid-derived EETs and PGs may in fact be the mediators of these responses\textsuperscript{32, 33}. These studies observed that pharmacological inhibition of EETs and PGs greatly inhibited retinal arteriole vasodilations during flicker stimulation in rodents. This hypothesis is yet to be investigated in humans. For Aim 3 of my doctoral project, I studied the effects of drugs that inhibit EETs and PGs on luminance flicker-induced retinal vasodilation in healthy people.

A poor understanding of how the retinal flicker response is affected by diabetes presents a major barrier to its routine evaluation in longitudinal clinical studies. One potentially important factor may be hyperglycaemia, which is recognised in humans to rapidly impair systemic endothelial function\textsuperscript{34-36} and reduce vascular elasticity\textsuperscript{37, 38}. Acute hyperglycaemia might similarly impair luminance flicker-induced retina vasodilation in people with diabetes. As these responses may be a function of retinal ganglion cell activity\textsuperscript{1}, the potential effects of hyperglycaemia on ganglion cell function should be considered. Furthermore, retinal vasodilation during flicker stimulation may be dependent on vascular elasticity, so this relationship warrants investigation. Of the two major forms of diabetes, type 1 diabetes represents the more homogeneous clinical phenotype. This form of diabetes arises from the destruction of the pancreatic islet $\beta$-cells responsible for insulin production. In contrast, type 2 diabetes is characterised by insulin resistance of an unclear aetiology, with some patients progressing to late stage $\beta$-cell loss. People with type 1 diabetes therefore represent the ideal population to investigate the relationship between blood glucose levels and retinal flicker responses. Consequently, I investigated changes in systemic arterial elasticity, retinal ganglion cell function and flicker light-induced vasodilation during euglycaemic and hyperglycaemic clamps in people with type 1 diabetes.

Acute vascular dysfunction during hyperglycaemia may involve the generation of oxidative stress\textsuperscript{24}. This suggests a potential role for antioxidants in the protection of retinal neurovascular function. Vitamin C (ascorbic acid) was previously found to prevent systemic arterial stiffening in non-diabetic people\textsuperscript{37} and endothelial dysfunction in non-diabetic and type 1 diabetic people\textsuperscript{39, 40} during acute hyperglycaemia. I therefore studied whether vitamin C could protect systemic
arterial elasticity, retinal ganglion cell function and luminance flicker-induced vasodilation during hyperglycaemia in people with type 1 diabetes.

Preliminary evidence suggests that the retinal flicker response may represent a sensitive marker of retinal damage in diabetes. However, before investigating chronic changes in longitudinal clinical studies, more information is needed on the major mechanisms and causes of acute changes in diabetes. Therefore, the overall purpose of this doctoral project was to understand why luminance flicker light-induced retinal vasodilation might be reduced in people with diabetes. To achieve this goal, I investigated the role of test conditions and arachidonic acid-derived signalling molecules in these responses; and the impact of acute hyperglycaemia and vitamin C treatment in people with type 1 diabetes.
1.2 Research aims

The aims and hypotheses of this PhD project were:

**Aim 1:** To investigate the effect of repeated testing on luminance flicker-induced retinal vasodilations in healthy people.

*Hypothesis:* Retinal vessel dilations during luminance flicker stimulation will be reduced after five but not 30 minutes of rest in healthy people.

**Aim 2:** To investigate the effect of ambient lighting on luminance flicker-induced retinal vasodilations in healthy people.

*Hypothesis:* Luminance flicker-induced retinal vasodilations will be equivocal between tests conducted under reduced and normal ambient lighting in healthy people.

**Aim 3:** To investigate the role of EETs and PGs in luminance flicker-induced retinal vasodilation in healthy people.

*Hypothesis:* Inhibition of EETs and PGs with oral fluconazole 400 mg and dispersible aspirin 600 mg, respectively, will reduce luminance flicker-induced retinal vasodilations in healthy people.

**Aim 4a:** To investigate the effects of euglycaemic and hyperglycaemic clamps on luminance-flicker induced retinal vasodilations, retinal ganglion cell function and systemic arterial elasticity in people with type 1 diabetes.

*Hypothesis:* Luminance flicker-induced retinal vasodilations and systemic arterial elasticity will be improved by euglycaemic clamp (glucose = 6 mmol/l) compared to baseline and impaired by hyperglycaemic clamp (glucose = 15 mmol/l) compared to euglycaemia in people with type 1 diabetes. Retinal ganglion cell function will be stable across glucose levels.

**Aim 4b:** To investigate the effect of antioxidant treatment prior to hyperglycaemic clamps on luminance flicker-induced retinal vasodilation, retinal ganglion cell function and systemic arterial elasticity in people with type 1 diabetes.

*Hypothesis:* Intravenous vitamin C 2 g will prevent impairments in luminance flicker-induced retinal vasodilations and systemic arterial elasticity between euglycaemic and hyperglycaemic clamps in people with type 1 diabetes. Vitamin C will not significantly affect retinal ganglion cell function.
1.3 Overview of the thesis

This thesis is organised into nine chapters. **Chapter 1** provides an introduction to the topic of flicker light-induced retinal vasodilation in diabetes research and describes the important gaps in the literature surrounding the factors potentially influencing this response. The specific aims of this doctoral project are described, as are the related hypotheses to be tested.

**Chapter 2** provides a detailed review of retinal neurocircuitry and the current state of knowledge of neurovascular coupling in the retina. It then describes the relevant literature on diabetes and its effects on flicker light-induced retinal haemodynamic responses. An overview of diabetes and DR is provided, including a description of important pathological features. This chapter concludes with a discussion of the role of oxidative stress in DR and the potential for antioxidant treatment to protect against impaired retinal vascular function during periods of hyperglycaemia.

In **Chapter 3**, the general methods used throughout this doctoral project are described. These methods are presented under common themes to minimise repetition. Differences in techniques between aims are outlined where applicable. Specific methodological details appear again in papers included *in lieu* of traditional thesis chapters.

**Chapters 4 to 7** report on the results of my four studies and associated aims. **Chapter 4** describes the results of my first study on whether luminance flicker-induced retinal vasodilation is affected by repeated testing. **Chapter 5** presents the results of my second study on whether these retinal flicker responses are affected by the level of ambient lighting. **Chapter 6** presents the findings of my third study on the role of EETs and PGs in these retinal flicker responses. These three chapters have been peer-reviewed and published. They are presented as they appear in their respective journals, with the exception of minor editing to maintain consistent spelling and figure, table and reference numbering within the context of the thesis. **Chapter 7** describes the results of my fourth and final study on whether euglycaemia and hyperglycaemia affect retinal and systemic vascular functions in type 1 diabetes, with or without vitamin C. This chapter is under review at the time of writing. It also includes supplementary analyses that were not submitted for publication.

In **Chapter 8**, a general discussion of my results is presented in the context of the current literature on luminance flicker light-induced retinal vasodilation in diabetes and my stated research aims. The important findings of my doctoral research are
highlighted and their potential scientific basis is explained. In addition, a discussion of the strengths and limitations of my doctoral research is provided.

Finally, in Chapter 9, a summary of the overall findings of my doctoral project is provided and the significance of my results to the broader research community is explained. The important research questions generated by my work are highlighted and potential avenues for future research are suggested.
CHAPTER 2. LITERATURE REVIEW
2.1 Introduction

Blood vessels in the retina dilate when stimulated with flickering light. This response reflects an increase in blood flow and is thought to be a function of ganglion cell activity. Flickering light can be in the form of luminance modulation, where the wavelength of the light is held constant, or chromatic modulation, where the wavelength of the light changes without a change in luminance. Flickering light may contain components of both luminance and chromatic flicker, in which case it is referred to as heterochromatic flicker. Both luminance and chromatic flicker stimuli affect vasodilations in retinal blood vessels. However, luminance and chromatic flicker are relatively specific for the magnocellular and parvocellular visual pathways, respectively, discussed in more detail below. The focus of this thesis was on luminance flicker-induced retinal vasodilation, which has been better studied in diabetic populations.

This chapter begins with a review of retinal neurocircuitry and a discussion of the way in which different characteristics of light affect different neuron populations. I have discussed the physiological properties of the main types of neurons, with a particular focus on the three main types of primate retinal ganglion cells: midget, parasol, and small bistratified ganglion cells. This information was fundamental to understanding how luminance flicker stimulation is converted to electrical signals and triggers an increase in retinal blood flow.

Knowledge of the physiological properties of retinal neurons is important to understand tests of retinal function. In the next section, the different types of clinical tests used to measure the function of different retinal neuron populations are discussed. Tests of retinal ganglion cell function were of particular interest to my doctoral project, given that increased blood flow may reflect ganglion cell activity. My review of retinal electrophysiology was especially important for the development of Aim 4, which included tests of ganglion cell function.

After my review of the physiological properties of individual neuron populations and their related clinical electrophysiological tests, a discussion of the haemodynamic changes induced by flicker stimulation is presented. The impact of different characteristics of flicker light on retinal haemodynamic changes, such as the luminance, wavelength, light adaptation and modulation depth, is reviewed in this section. Aims 1 and 2 specifically addressed whether luminance flicker-induced retinal vasodilations are reduced by light adaptation during repeat testing or smaller modulation depths under
higher ambient lighting, respectively. In addition, I have discussed the potential neuronal basis of haemodynamic changes with different forms of flicker light stimulation. The information in this section was fundamental to all the Aims of this doctoral project.

Signalling molecules are the link between neuronal activity and haemodynamic changes in the retina. In the next section, I have reviewed the main signalling molecules hypothesised to mediate flicker-induced retinal vasodilation: nitric oxide, arachidonic acid metabolites and vasoactive metabolites. Prior to this doctoral project, no study had investigated the potential role of arachidonic acid metabolites in these responses in humans. Aim 3 specifically addressed whether these molecules are important under healthy circumstances and provided information on the likely molecular pathways altered in diabetes.

The next sections of this literature review present an overview of diabetes and DR. I have discussed the biochemistry of diabetic complications at a general level and the cellular changes that occur in the retina during DR. An understanding of the biochemistry of diabetic complications was especially important for the final aims of my doctoral project. Observations on luminance flicker-induced retinal vasodilations, neuronal function and systemic arterial elasticity in people with diabetes are then discussed. These three functional markers were studied in people with type 1 diabetes in Aim 4 of this doctoral project.

Chronic hyperglycaemia in diabetes causes progressive damage (over years) to blood vessels in the eyes and other locations. However, as this review shows, vascular function can fluctuate dramatically in response to glucose normalisation or hyperglycaemia in diabetes. It is therefore unclear whether DR might be related to periods of impaired vascular function with higher blood glucose levels. To address this gap in the literature, Aim 4a described changes in luminance flicker-induced vasodilation, retinal ganglion cell function and systemic arterial elasticity during euglycaemia and hyperglycaemia in people with type 1 diabetes.

To conclude, this literature review presents a discussion of the potential role for antioxidants in diabetic complications. Biochemical evidence suggests that hyperglycaemia may contribute to diabetic complications by increasing oxidative stress. Therapies aimed at reducing oxidative stress might therefore improve retinal neuronal or vascular function during hyperglycaemia. Previous studies have found benefits with vitamin C on systemic vascular function. Aim 4b reports on
whether similar benefits may be expected for retinal vascular function in people with type 1 diabetes.

Luminance flicker-induced retinal vasodilation is a potential sensitive marker of retinal neuronal or vascular dysfunction in diabetes. However, my review of the current literature has highlighted several important gaps in our understanding of this response. The Aims of my doctoral project were designed to address these gaps and help explain why luminance flicker-induced retinal vasodilation is impaired in people with diabetes.

2.2 Retinal Neurocircuitry

Light received at the back of the eye is converted to an electrical signal by the retina and transmitted to the brain. This is accomplished by way of a rapid series of electrochemical changes in highly specialised retinal neurons. Signals from the retina are sent to the lateral geniculate nucleus (LGN) and from there to the primary visual cortex. Higher order processing is then used to obtain conscious visual information.

Anatomically, the retina lies between the transparent vitreous body and the vascular choroid. It is subdivided into ten anatomical layers, which from outer to inner are the: non-sensory retinal pigment epithelium (RPE); layer of rods and cones; outer limiting membrane; outer nuclear layer; outer plexiform layer; inner nuclear layer; inner plexiform layer; ganglion cell layer; nerve fibre layer and the inner limiting membrane (Figure 1). These layers contain the principal retinal neurons: photoreceptors; bipolar cells; ganglion cells; horizontal cells and amacrine cells. Other non-neuronal cells provide additional support, including glial cells (Müller cells and astrocytes) and vascular cells (pericytes and endothelial cells).
Figure 1. Schematic cross-section of the retina (not to scale). A, amacrine cells; B, bipolar cells; C, cones; G, ganglion cells; H, horizontal cells; P, photoreceptor cell bodies; R, rods; RPE, retinal pigment epithelium.

2.2.1 Photoreceptors

Photoreceptors are classified as either rods or cones. Rods provide low acuity, monochromatic vision under low-light (scotopic) conditions. In contrast, cones mediate high acuity colour vision at high (photopic) light intensities. Phototransduction occurs within the free ends of photoreceptors, which reside in the layer of rods and cones. The cell bodies of rods and cones are found in the outer nuclear layer and their axons synapse with other neurons in the outer plexiform layer.

Rods contain the photosensitive pigment rhodopsin\textsuperscript{45}. Rhodopsin is a G protein-coupled receptor that consists of a chromophore, 11-cis retinal, linked to the protein opsins. Absorbed photons change 11-cis retinal to all-trans retinal, which activates rhodopsin and leads to a reduction in cytosolic cyclic guanosine monophosphate
(cGMP). In darkness, cGMP keeps cation channels open and allows an influx of Na$^+$, Ca$^{2+}$ and Mg$^{2+}$ ions to maintain the cell in a partly depolarised state. This provides a steady release of glutamate at the synaptic terminal. In light, reduced cGMP levels trigger the closure of cation channels and a reduction in glutamate release.

The primate visual system contains three types of cones, differentiated by the wavelength sensitivities of their visual pigments. Human cone pigments are sensitive to long (560 nm; red), medium (530 nm; green) and short (420 nm; blue) wavelengths and are found in L-, M- and S-cones, respectively$^{45}$. In an average human trichromat, roughly 50% of cones are L-cones, 40% are M-cones and the remaining 10% are S-cones$^{46}$. Light signals in cones are processed in a similar manner to rods, as described above. **The wavelength of the luminance flicker stimulus used in my doctoral project was in the green spectrum and was therefore selective for M-cones.**

Photoreceptors synapse with the dendrites of bipolar and horizontal cells at rod spherules and cone pedicles$^{47}$. Rod spherules contain a single invaginating ribbon, whereas each cone pedicle contains 20 to 50 such ribbons. Rod spherules generally contact two horizontal cells laterally and one to three rod bipolar cells centrally. Horizontal and ON cone bipolar cells form invaginating contacts with cone pedicles, while OFF cone bipolar cells contact the pedicle base. Many types of bipolar cells are in contact with each cone pedicle.

### 2.2.2 Bipolar cells

Bipolar cells are intermediary neurons between the photoreceptors and ganglion cells. There are at least 13 types of bipolar cells, including one rod bipolar cell and 12 cone bipolar cells (nine diffuse bipolar cells; two midget bipolar cells and one blue bipolar cell)$^{48}$. Bipolar cells can be further classified as ON or OFF depending on their response to light. ON bipolar cells have inhibitory glutamate receptors at their photoreceptor synapses and are therefore depolarised by light. In contrast, OFF bipolar cells express excitatory glutamate receptors and are hyperpolarised by light. The axons of ON bipolar cells terminate in the inner part of the inner plexiform layer, while those of OFF bipolar cells terminate in the outer part.

All rod bipolar cells are ON cells. Rod bipolar cells do not contact ganglion cells directly, but send their signals through AII amacrine cells to cone bipolar cells$^{48}$. Diffuse cone bipolar cells non-selectively contact five to ten L- and M- cones, with occasional input from S-cones$^{47}$. Midget cone bipolar cells synapse with a single L- or M-cone. At the fovea, their density is such that midget bipolar cells form one-to-one
connections with midget ganglion cells. This allows a single cone to provide input to a single nerve fibre, which is important for high acuity vision. Blue cone bipolar cells selectively contact one to five S-cones and are all ON cells.

2.2.3 Ganglion cells

Ganglion cells are third-order retinal neurons. They receive signals from bipolar and amacrine cells in the inner plexiform layer and project through the optic nerve to the LGN. Roughly 15 different types of ganglion cells are present in primates. Each occupies a different level of the ganglion cell layer to allow their dendrites to cover the retina without interference from one another. Ganglion cells are specialised to process particular features of a light stimulus, such as its size, wavelength, luminance or direction.

The receptive fields of ganglion cells are classically described as having a central region and a concentric antagonistic surround. In general, ganglion cells are depolarised by stimuli that are brighter (ON centre/OFF surround) or darker (OFF centre/ON surround) than the background (Figure 2). ON centre ganglion cells receive signals from ON bipolar cells in the inner plexiform layer, while OFF centre ganglion cells receive signals from OFF bipolar cells. The behaviour of receptive field centres can be readily explained by the bipolar cell inputs to ganglion cells. However, the mechanisms of surround inhibition are less well understood. Feedback inhibition from horizontal cells appears to be important in primates, although inner retinal contributions have also been suggested from work in other mammals.

I speculated that luminance flicker stimulation, as used in this doctoral project, would preferentially stimulate ON and OFF centre ganglion cells during the ON and OFF flicker phases, respectively.

Ganglion cells can be further distinguished on the basis of their spatial summation. Early studies in cats identified ganglion cells with linear and non-linear summation to grating stimuli, respectively termed X and Y cells. Cone inputs to midget ganglion cells and S-cone inputs to small bistratified ganglion cells are summed linearly. However, these cells have very different morphological and functional characteristics from cat X cells. Shapley and Perry argued that parasol ganglion cells could be subdivided into those with linear (X-like) and non-linear (Y-like) spatial summation. However, direct recordings suggest that all parasol cells exhibit some degree of non-linear summation. A frequency-doubled response is evident both with
red-green chromatic flicker and stationary contrast-reversal stimuli. The mechanism is unclear but may involve diffuse bipolar cells.

Parasol ganglion cells
ON centre (left), OFF centre (right)

Midget ganglion cells
Green centres/red surrounds
ON centre (left), OFF centre (right)

Midget ganglion cells
Red centres/green surrounds
ON centre (left), OFF centre (right)

Small bistratified ganglion cells
Spatially coextensive blue ON and yellow OFF receptive fields

**Figure 2.** Receptive field organisation of the three main types of retinal ganglion cells.

*Midget ganglion cells*

The dominant anatomical type of ganglion cell in primates is the midget cell. This one type comprises 80% of the total ganglion cell population. Midget ganglion cells are either OFF or ON and stratify in the outer and inner plexiform layer, respectively. OFF cells occur at a rate of approximately five for every three ON cells to account for their smaller dendritic fields. Midget cells have relatively small dendritic and receptive field areas compared to other ganglion cells. They are densely represented.
at the fovea, where they transmit signals from a single L- or M-cone to the LGN\textsuperscript{58, 59}. The high density of midget cells near the fovea is important for high acuity central vision.

Both ON and OFF midget ganglion cells receive roughly half of their synaptic connections from midget bipolar cells and half from amacrine cells near the fovea\textsuperscript{58}. This wiring makes them sensitive to light of red and green wavelengths. Furthermore, their receptive field centres and surrounds receive selective and opposing input from L- and M-cones. This translates to four arrangements: red ON centres with green OFF surrounds; red OFF centres with green ON surrounds; green ON centres with red OFF surrounds; and green OFF centres with red ON surrounds\textsuperscript{60, 61}. In addition, some midget cells might receive mixed L- and M-cone surround inputs\textsuperscript{51}.

The axons of midget ganglion cells project to the parvocellular layers three through six of the dorsal LGN\textsuperscript{62}. Parvocellular neurons in the LGN in turn project to layer 4Cβ of the primary visual cortex in the occipital lobe\textsuperscript{63}. This cortical layer appears to be exclusively innervated by parvocellular neurons. The parvocellular pathway forms the basis of red-green colour vision because of its antagonistic L- and M-cone input to midget ganglion cells. After the primary visual cortex, visual information is further processed by higher order temporal and parietal neurons\textsuperscript{64}.

Midget cells have physiological properties distinct from other ganglion cells. They exhibit sustained (tonic) responses when continuously illuminated by wavelengths near their peak sensitivity\textsuperscript{65}. With luminance flicker, these cells respond poorly at frequencies below 10 Hz and cone contrasts below 20%\textsuperscript{11}. Red-green chromatic flicker evokes strong responses in midget cells at frequencies between 1-20 Hz with cone contrasts as low as 5%. However, their sensitivity declines sharply above 20 Hz. Their response to red-green flicker is maximal with stimulation of both their receptive field centres and surrounds due to their selective connections to L- and M-cones. The ability of human observers to perceive red-green flicker falls away at frequencies above 2 Hz, despite physiological support from midget cells to detect higher frequencies. Luminance flicker-induced retinal vasodilation is unlikely to be mediated by midget cells, although a small contribution cannot be excluded.

\textit{Parasol ganglion cells}

The second most common type of ganglion cell in primates, at 10\% of the total population, is the parasol cell\textsuperscript{57}. Parasol cells are larger than their midget cell
counterparts and are similarly concentrated towards the fovea\textsuperscript{66, 67}. They differ from midget cells in that they do not have an even distribution of synapses from bipolar and amacrine cells. ON parasol cells receive less than 20\% of their synaptic connections from bipolar cells\textsuperscript{68}, while roughly 30\% of OFF parasol cell synapses are from bipolar cells\textsuperscript{69}. The receptive fields of parasol cells lack colour specificity due to mixed L- and M-cone inputs to diffuse bipolar cells\textsuperscript{60}. Their receptive fields are therefore simply ON centre/OFF surround or OFF centre/ON surround.

Parasol cell axons carry luminance signals to magnocellular neurons in the ventral two layers of the LGN\textsuperscript{62}. These neurons in turn project to layer 4C\textsubscript{α} of the primary visual cortex, an area that appears to be exclusive for the magnocellular pathway\textsuperscript{62}. Parasol cells are sensitive to luminance contrast and are probably important for gross object discrimination\textsuperscript{11}.

The physiological properties of parasol cells are quite different to midget cells. Parasol cells display transient (phasic) responses with continuous illumination\textsuperscript{65}. They are very sensitive to luminance flicker; peak sensitivities occur around 10 Hz with a mere 3.5\% cone contrast\textsuperscript{11}. Parasol cells show virtually no response to chromatic flicker at the fundamental frequency. However, a response may be observed at twice the flicker frequency with red-green flicker due to the non-linear summation of L- and M-cone signals\textsuperscript{11, 53}. Similar frequency-doubled responses may be observed with stationary black and white square reversals\textsuperscript{56}. Responses are generally larger with flicker stimuli that cover their receptive field centres and surrounds\textsuperscript{11}. Luminance flicker detection thresholds in humans closely parallel physiological data for parasol cells, with peak sensitivities at frequencies around 10 Hz. The high sensitivity of parasol cells to luminance flicker suggests that these ganglion cells may be important neuronal mediators of retinal vasodilation with this stimulus.

Small bistratified ganglion cells

A third important recently discovered ganglion cell is the small bistratified ganglion cell. These cells comprise about 3\% of the ganglion cell population near the fovea\textsuperscript{70}. Small bistratified cells are similar in size to parasol cells but have two dendritic layers in the inner and outer parts of the inner plexiform layer\textsuperscript{71}. The inner layer of dendrites receives an ON signal from blue bipolar cells, while the outer layer receives an OFF signal from diffuse bipolar cells coupled to L- and M-cones\textsuperscript{72}. Small bistratified cells receive roughly 40\% of their inputs from blue bipolar cells at their inner dendritic
layer and 20% of their inputs from diffuse OFF bipolar cells at their outer layer\textsuperscript{70}. Amacrine cells supply 40% of inputs to the small bistratified cell, distributed evenly between each layer. Small bistratified cells have spatially coextensive blue ON and yellow OFF receptive fields that, unlike midget and parasol cells, appear to lack an antagonistic surround\textsuperscript{70}.

The axons of small bistratified ganglion cells project to koniocellular neurons in the interlaminar zones of the LGN\textsuperscript{73}. Koniocellular neurons then project to layers 4A and 2/3 of the primary visual cortex\textsuperscript{63}. This pathway forms the basis of blue-yellow colour-opponency and, with the parvocellular pathway, appears to be an important contributor to primate trichromatic vision.

Less is known about the physiological properties of small bistratified ganglion cells compared with midget and parasol cells. The phasic blue ON cells described by Lee and colleagues\textsuperscript{11} probably represent small bistratified ganglion cells. These cells show weak responses to luminance flicker at frequencies below 10 Hz and virtually no response with red-green flicker. Blue ON cells respond well to blue-green flicker at frequencies between 1-20 Hz with similar sensitivities to midget cells with red-green flicker. Blue-yellow chromatic flicker may have been more optimal for these cells, given that diffuse OFF bipolar cells provide mixed L- and M-cone signals to their outer dendritic layer\textsuperscript{72}. The ability of human subjects to detect blue-green flicker declines rapidly at frequencies above 2 Hz, as is the case for red-green flicker\textsuperscript{11}. The relatively low numbers of small bistratified ganglion cells and physiological similarities with midget ganglion cells suggest that these cells are not major contributors to luminance flicker-induced retinal vasodilation.

2.2.4 Horizontal cells

The cell bodies of horizontal cells are located in the inner nuclear layer. Their dendrites project to the outer plexiform layer, where they form lateral contacts in the invaginations of rod spherules and cone pedicles. Horizontal cells sum the input from neighbouring photoreceptors and provide feedback inhibition at the photoreceptor synapse. The mechanism of inhibition is not clear but several hypotheses have been proposed: release of the inhibitory neurotransmitter \(\gamma\)-aminobutyric acid (GABA); hemigap junctions between horizontal cells and photoreceptors; and modulation of Ca\textsuperscript{2+} channels at the presynaptic terminal\textsuperscript{47}. Horizontal cells of the same type are coupled
together by gap junctions. This allows their receptive fields to extend beyond the limit of their dendrites.

Two types of horizontal cells are present in primates, designated H1 and H2 cells. H1 cells contact L- and M-cones and rarely contact S-cones. In contrast, H2 cells preferentially contact S-cones and less frequently contact the other cone types. Both H1 and H2 cells are hyperpolarised by light and neither exhibits colour opponency. The absence of a colour-opponent mechanism indicates that horizontal cells alone are not sufficient for colour vision in primates. Interestingly, H1 cells show a small frequency-doubled response with isoluminant red-green light exchange, which suggests a degree of non-linear summation of cone inputs.

2.2.5 Amacrine cells

Amacrine cells are important interneurons in the inner retina. Most have their cell bodies in the inner nuclear layer, although some are displaced to the ganglion cell layer. Their dendrites synapse with bipolar cells, ganglion cells and other amacrine cells. Upward of half of all inputs to ganglion cells are supplied by amacrine cells.

About 30 different types of amacrine cells are recognised in mammals and their functions are not all understood. Most, if not all, neurotransmitters are expressed within their total population.

Amacrine cells are traditionally grouped into two main types. Diffuse cells branch vertically through the inner plexiform layer, while the processes of stratified cells are predominantly located within a single plane. Neurotransmitter expression can help to further classify these cells. For instance, glycine is found in half of all amacrine cells and of these, all are diffuse cells.

The most common type of amacrine cell is the AII cell, a diffuse glycinergic cell that makes up about 12.5% of the total population. These cells have two strata of dendrites in the inner and outer parts of the inner plexiform layer and are best known for their role in the transmission of rod signals. Rod bipolar cells form excitatory contacts with AII cells in the inner part of the inner plexiform layer. In turn, AII cells form gap junctions with ON cone bipolar cells in the inner part of the inner plexiform layer and inhibitory glycinergic synapses with OFF cone bipolar cells in the outer part of this layer. The net effect of these connections is depolarisation of ON ganglion cells and hyperpolarisation of OFF ganglion cells during rod illumination.

The most studied type of stratified amacrine cell is the starburst cell, which comprises roughly 10% of all amacrine cells. These cells are found in equal numbers
on either side of the inner plexiform layer. Starburst cells in the inner nuclear layer stratify in the outer part of the inner plexiform layer, whereas those in the ganglion cell layer stratify in the inner part\textsuperscript{76}. Interestingly, starburst cells release both acetylcholine and GABA, which gives them both excitatory and inhibitory actions\textsuperscript{79}. These dual actions are thought to mediate the activity of direction-sensitive ganglion cells\textsuperscript{47}. Direction-sensitive ganglion cells respond optimally to movement in a preferred direction, but respond minimally to movement in the opposite direction.

\textbf{2.3 Electroretinography}

Knowledge of the physiological properties of retinal neurons makes it possible to test their function in living human subjects. These techniques use electrical recordings from the retina during light stimulation and fall under the broad domain of electroretinogram (ERG) studies. These studies, though an indirect measure of neuronal function, can be used to infer information about the health of neurons in different parts of the retina separated by neuron type or location.

The full-field (Ganzfeld) ERG measures the summed response of retinal neurons to flashes of spatially uniform luminance\textsuperscript{80, 81}. Different parameters are used to selectively stimulate rods, cones or both photoreceptor types. Tests are performed in the dark, light or specific chromatically-adapted states with flash strengths ranging from 0.01-30.0 cd*s/m\textsuperscript{2}. Standard ERG protocols also include measurement of the light-adapted 3.0 cd*s/m\textsuperscript{2} 30 Hz flicker response. This flicker response is specific for cones as rods are unable to respond to frequencies this high. Signals are recorded from electrodes positioned on or around the eye. The typical full-field ERG response consists of an initial negative a-wave followed by a larger positive b-wave\textsuperscript{80, 81}. The a-wave reflects photoreceptor function, while the b-wave is primarily due to ON bipolar cell depolarisation. The ascending b-wave contains a series of three to four oscillatory potentials (OPs). These oscillations can be distinguished with a band-pass filter of approximately 100-300 Hz and are probably derived from the bipolar or amacrine cells\textsuperscript{82}. A late negative response occurs after the b-wave in the light-adapted state, termed the photopic negative response. Its attenuation by experimental glaucoma or tetrodotoxin strongly suggests that it depends on ganglion cell function\textsuperscript{83}.

The multifocal ERG (mfERG) is a related technique used to measure light-adapted responses at many discrete retinal locations\textsuperscript{84}. It is used clinically to identify specific areas of neuronal dysfunction. The stimulus is an array of usually 61 or 103
hexagonal elements, each with a 50% chance of illumination per frame and the
sequence of transitions for each square pseudo-independent of all of the others. The
pattern is displayed onto the central retina. The luminance contrast between light and
dark elements should be at least 90%. Local mfERG signals are calculated by
correlating the continuous recorded ERG signal with the sequence of ON and OFF
phases for each element. Electrode arrangements are identical to the standard ERG.
Each signal consists of an initial negative component (N1) and positive peak (P1),
usually followed by a second negative trough (N2). It is thought that N1 reflects cone
function, while P1 and N2 represent bipolar or amacrine cell activity. The mfERG is
useful to detect local functional abnormalities but is inferior to the full-field ERG for
generalised changes.

The PERG is a technique used to investigate the function of inner retinal
neurons, primarily ganglion cells, in the central retina. It involves stimulation of the
macula with gratings or checkerboards in which the pattern alternates while holding the
overall luminance constant. At frequencies below 3 Hz, transient electrical responses
are observed between each contrast reversal. The normal transient response consists of a
small negative component near 35 milliseconds (N35), a large positive component near
50 milliseconds (P50) and a large negative component near 95 milliseconds (N95)
(Figure 3). The P50 component is thought to reflect ganglion cell activity but may also
be driven by bipolar or amacrine cells. Ganglion cell receptive field centre and surround
antagonism with contrast reversals may give rise to the N95 component. At faster
frequencies, such as 8 Hz and above, steady-state responses are observed. These
responses are approximately sinusoidal with an amplitude similar to the transient N95
component and a frequency twice that of the stimulus. Interestingly, parasol ganglion
cells exhibit frequency-doubled responses with chromatic flicker and stationary contrast
reversals with optimal frequencies around 10 Hz. This suggests that parasol cells
may provide the physiological basis of the PERG steady-state response. Potential
cchanges in components of the PERG transient and steady-state responses with
euglycaemia and hyperglycaemia were studied in Aim 4.

Other standard electrophysiology tests include the visual evoked potential (VEP)
and the electrooculogram. The VEP is obtained from the occipital scalp overlying the
primary visual cortex. These studies require good central vision and are dependent on
intact post-retinal pathways. The electrooculogram measures electrical potentials across
the RPE during dark and light adaptation and can be affected by diseases of the outer retina.

![Graph showing PERG transient response with black and white contrast reversals](image)

**Figure 3.** Typical PERG transient response with black and white contrast reversals.

### 2.4 Flicker-evoked retinal haemodynamic changes

Activation of retinal neurons during flicker stimulation is accompanied by an increase in blood flow. Initial evidence that light affects retinal neuronal activity was found by studies of glucose metabolism in cynomolgus monkeys\(^87\). Constant light reduced glucose metabolism in the outer retina, whereas flickering light increased glucose metabolism in the inner retina. These findings are consistent with photoreceptor inactivation by constant light and ganglion cell activation by flickering light. It was subsequently observed by laser Doppler flowmetry in cats that diffuse luminance flicker increases blood flow and oxygen consumption in the optic nerve\(^88\). Blood flow increases rapidly in the first few seconds and quickly returns to baseline once the stimulus is stopped. Optic nerve blood flow during diffuse luminance flicker follows a similar time course in humans\(^89\).

The degree of retinal hyperaemia during luminance flicker is dependent on the properties of stimulus. Larger increases in blood flow are induced with more intense stimuli or to a fixed stimulus after a period of dark adaptation\(^88\). **These observations were of particular relevance to Aim 1:** if larger increases in blood flow occur after
dark adaptation, smaller responses might occur with repeated stimulation if light adaptation occurs during the test period.

Larger responses are observed as the modulation depth, defined as the ratio of the illuminance modulation amplitude to the mean illuminance, is increased towards 100%. The modulation depth of luminance flicker can be controlled by varying the luminance of the stimulus light during the ON and OFF flicker phases. The effective modulation depth may also be altered by light from the environment. For example, higher ambient lighting might reduce the difference between the ON and OFF flicker phases and cause a smaller increase in blood flow. **Aim 2 investigated whether higher ambient lighting reduces the magnitudes of luminance flicker-induced retinal vasodilations.**

Equivalent responses are observed with red or green wavelengths at photopic intensities. However, rod sensitivities may become important at lower intensities. Optic nerve hyperaemia follows a band-pass function of the stimulus frequency and is maximal at frequencies near 10 Hz. Luminance flicker at this frequency is relatively selective for parasol ganglion cells, although midget cells may also be stimulated at high luminance contrasts.

Red-green chromatic flicker similarly induces optic nerve hyperaemia, although responses may be 40% smaller than with luminance stimuli of red or green wavelengths. Haemodynamic responses to chromatic flicker are optimal at frequencies near 2 Hz and decline sharply above 10 Hz, in contrast to luminance flicker. This suggests the involvement of midget ganglion cells in red-green flicker-induced optic nerve hyperaemia.

Blood flow in the retina is somewhat harder to measure than in the optic nerve. One study combined measurements of laser Doppler velocimetry and vessel diameters to estimate flow in healthy volunteers, given that flow through a vessel is proportional to the mean velocity and square of the vessel diameter. Green luminance flicker at 8 Hz for 60 seconds increased arteriole flow by 59% and venule flow by 53%. These changes were largely attributable to increased blood velocities, which led the authors to conclude that flicker-induced haemodynamic changes probably originate in the small arterioles.

Reliable estimates of retinal blood flow are difficult to obtain due to the high variability of blood velocity measurements. However, in the absence of blood velocities, changes in vessel diameters during flicker stimulation remain a useful proxy
for retinal hyperaemia. Flicker light-induced retinal vasodilation in healthy people has been studied in near-real time with the DVA system (IMEDOS, Jena, Germany) in several studies, summarised in Table 1, with various stimulus characteristics and techniques. This doctoral project used the DVA system to investigate real time changes in retinal arteriole and venule dilations with green light and a flicker frequency of 12.5 Hz.

Polak and colleagues\textsuperscript{41} measured vessel diameters continuously with a system similar to the DVA during 60 seconds of luminance flicker at various frequencies in healthy subjects. At 8 Hz, they observed dilations in the proximal arterioles and venules of 3.5% and 1.9%, respectively. Limitations of this study were a small sample of nine subjects and a low contrast stimulus with a minor chromatic component. Chromatic flicker also elicits retinal vessel dilations: red-green flicker at 12 Hz for 30 seconds induced dilations of 3.2% in arterioles and 4.9% in venules, respectively\textsuperscript{42}. Corresponding dilations for blue-green flicker were 2.6% and 3.4% for arterioles and venules, respectively. Isoluminant chromatic flicker induced smaller dilations than luminance flicker, which is consistent with studies of optic nerve blood flow. Furthermore, blue-green flicker may have induced smaller dilations than red-green flicker due to less specificity for midget ganglion cells. Examples of typical relative dilations in human retinal arterioles and venules during 20 seconds of 12.5 Hz luminance flicker are illustrated in Figure 4.

ERG studies indirectly support a link between retinal neuronal activity and haemodynamic responses. The amplitude of the flicker ERG frequency-doubled response is strongly correlated with optic nerve hyperaemia with both luminance and chromatic flicker stimuli\textsuperscript{91}. The flicker ERG frequency-doubled response, while less specific than the PERG, is thought to have an important inner retinal component. It is difficult to study retinal haemodynamic changes with typical PERG stimuli. However, one study reported a 38% increase in optic nerve blood flow with isoluminant black and white square reversals at 8 Hz in one volunteer\textsuperscript{93}. This stimulus is used in PERG studies to measure the ganglion cell-derived steady-state response.

\section*{2.5 Pharmacology of flicker-evoked responses}

Retinal neuronal activity triggers the release of various vasoactive signalling molecules. These may be released directly from neurons themselves or from other supportive cells, such as glia or endothelial cells. The three main groups of signalling
molecules hypothesised to mediate flicker light-induced retinal hyperaemia are nitric oxide, arachidonic acid metabolites and vasoactive by-products of metabolism. Potassium siphoning by glial cells was thought to be another potential mechanism, but observations in rodents have not supported this hypothesis in the retina\textsuperscript{94}.

2.5.1 Nitric oxide

Nitric oxide is a ubiquitous mediator of vasodilation throughout the body. It relaxes smooth muscle cells by activating guanylate cyclase, which then activates protein kinase G and reduces intracellular calcium levels. NOS is the enzyme responsible for nitric oxide production, of which there are three variants in humans. eNOS and nNOS are calcium-dependent forms important for cell signalling. Inducible NOS (iNOS) is a calcium-independent form involved in immune responses. The calcium-dependent forms are likely to mediate flicker-evoked retinal haemodynamic responses under normal conditions.

![Graph showing relative dilation of arterioles and venules in response to 12.5 Hz flicker](image)

**Figure 4.** Typical human retinal arteriole and venule relative dilations in response to 20 seconds of 12.5 Hz luminance flicker.

An important role for nitric oxide in retinal hyperaemia during flicker stimulation was identified early from animal and human studies. Systemic inhibition of NOS with \(\text{N}^6\)-nitro-L-arginine methyl ester (L-NAME) markedly attenuated optic nerve hyperaemia in cats during diffuse luminance flicker\textsuperscript{5, 6}. Basal optic nerve blood flow was also reduced, highlighting the importance of nitric oxide to normal retinal vascular tone. Similar results were found in humans during infusions with another NOS
inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA). Systemic NOS inhibition markedly attenuated retinal arteriole and venule dilations during diffuse luminance flicker. The inhibitors used in these studies were not specific for any particular form of NOS, which makes it difficult to draw conclusions on the cellular sources of nitric oxide.

Recent evidence suggests that nNOS is the dominant form of NOS in the retina under normal conditions. eNOS is roughly half as prevalent and is mostly restricted to blood vessels. Furthermore, flickering light triggers a dramatic increase in nitric oxide levels in the inner plexiform layer, which could be a result of ganglion, amacrine or bipolar cell production. Flicker-evoked retinal hyperaemia could therefore be due to local nitric oxide production in the inner retina with local diffusion to neighbouring blood vessels.

**Ganglion cells**

Ganglion cells express N-methyl-D-aspartate (NMDA) and non-NMDA ionotropic glutamate receptors and respond to agonists at both types of receptors. These observations, combined with strong bipolar cell immunoreactivity for glutamate, strongly suggest that glutamate is the main neurotransmitter that mediates ganglion cell activity in response to light stimuli.

NMDA receptors mediate nitric oxide synthesis in the central nervous system and may do the same in the retina. Ganglion cells contain nNOS and produce nitric oxide in response to stimulation with glutamate and NMDA. Glutamate binding to NMDA and non-NMDA receptors opens non-specific cation channels. However, NMDA receptors are more permeable to calcium ions than sodium or potassium ions. The ion concentration gradient determines the direction of flow: calcium and sodium are more concentrated outside the cell, whereas potassium is more concentrated in the intracellular fluid. An increase in intracellular calcium stimulates nNOS to produce nitric oxide. As may be expected, glutamate increases intracellular calcium levels in retinal ganglion cells and this increase is predominantly via NMDA receptors. Therefore, ganglion cells may produce nitric oxide in response to light stimulation and pre-synaptic glutamate release.

**Amacrine cells**

Another potential source of nitric oxide production during luminance flicker stimulation may be amacrine cells. Similar to ganglion cells, functional NMDA receptors are present in a sub-population of amacrine cells. Furthermore, nNOS has consistently been identified in studies of mammalian amacrine cells. Regardless of
whether amacrine cells are as physiologically sensitive to flicker stimuli as ganglion cells, their many synaptic connections with bipolar and ganglion cells could facilitate NMDA receptor-mediated nitric oxide production during flicker light stimulation.

**Table 1.** Studies of flicker-induced retinal vasodilation in healthy humans with the Dynamic Vessel Analyzer.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Stimulus</th>
<th>Increase (mean [SD])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polak K et al.(^{41})</td>
<td>Diffuse red-free luminance flicker for 60 seconds (various frequencies)</td>
<td>Proximal arterioles 3.5% (2.3%) (8 Hz)</td>
</tr>
<tr>
<td>(n = 9)</td>
<td></td>
<td>Proximal venules 1.9% (1.2%) (8 Hz)</td>
</tr>
<tr>
<td>Nagel E and Vilser W(^{111})</td>
<td>Diffuse green luminance flicker at 12.5 Hz for 20 seconds</td>
<td>Arterioles 7.4% (2.4%)</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kotliar KE et al.(^{42})</td>
<td>Diffuse red-green and blue-green chromatic flicker at 12 Hz (results are for 30 seconds duration)</td>
<td>Arterioles 3.2% (1.5%) (red-green flicker)</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td>Venules 4.9% (1.8%) (red-green flicker)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Garhofer G et al.(^{92})</td>
<td>Diffuse red-free luminance flicker at 8 Hz for 60 seconds</td>
<td>Arterioles ((n = 8)) 3.8% (1.6%)</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
<td>Venules ((n = 9)) 2.8% (1.2%)</td>
</tr>
<tr>
<td>Nagel E et al.(^{30})</td>
<td>Diffuse green luminance flicker at 12.5 Hz for 20 seconds</td>
<td>Arterioles 3.6% (2.9%)</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
<td>Venules 4.9% (2.0%)</td>
</tr>
<tr>
<td>Nguyen TT et al.(^{31})</td>
<td>Diffuse green luminance flicker at 12.5 Hz for 20 seconds</td>
<td>Arterioles 3.9% (1.8%)</td>
</tr>
<tr>
<td>(n = 33)</td>
<td></td>
<td>Venules 5.4% (2.1%)</td>
</tr>
</tbody>
</table>

### 2.5.2 Arachidonic acid metabolites

Another potential mechanism of flicker-evoked retinal hyperaemia is the release of vasodilatory arachidonic acid metabolites from glial cells, specifically EETs and PGs (Figure 5). The hypothesis is that activated ganglion cells release adenosine triphosphate (ATP) onto glial cells, which triggers a rise in intracellular calcium, activation of phospholipase A\(_2\) and the release of arachidonic acid from the cell membrane. Arachidonic acid is then converted to EETs and prostaglandins PGs within glial cells by cytochrome P450 (CYP) and cyclooxygenase (COX) enzymes, respectively\(^3\). EETs and PGs mediate smooth muscle relaxation by opening potassium ion channels.
channels, which hyperpolarises the cell and blocks calcium influx through voltage-gated calcium channels.

**Figure 5.** Hypothetical mechanism of flicker light-induced retinal vasodilation. AA, arachidonic acid; ATP, adenosine triphosphate; COX, cyclooxygenase; CYP, cytochrome P450; EETs, epoxyeicosatrienoic acids; P2, ATP receptor; PGs, prostaglandins; PLA\(_2\), phospholipase A\(_2\). Adapted from Attwell D et al.\(^3\).

The arachidonic acid hypothesis has received recent support from rodent studies. Inhibitors of EETs attenuated flicker-evoked retinal arteriole dilations in *ex vivo* rat retinas perfused with 95% oxygen\(^{32}\). Although inhibitors of PGs did not reduce flicker-evoked arteriolar dilations under hyperoxic conditions, this finding was observed when retinas were perfused with atmospheric levels of oxygen\(^{33}\). Dilations were blocked by the inhibition of neuron to glia signalling and replicated by direct glial stimulation, which suggests an important role for glial cells in these haemodynamic responses\(^{32}\). Interestingly, higher background levels of nitric oxide tended to produce arteriole constriction during flicker stimulation, although it is unclear if this finding was related to the high levels of oxygen used.

The *in vivo* production of EETs and PGs can be inhibited by fluconazole and aspirin, respectively. Fluconazole, an anti-fungal drug, is a potent inhibitor of CYP2C enzymes\(^{112}\). Fluconazole was previously used to demonstrate EET involvement in the control of radial artery tone and brachial artery flow-mediated dilation in humans\(^{113, 114}\). Aspirin, an irreversible inhibitor of COX enzymes\(^{115, 116}\), has similarly been used to
show that PGs can mediate cutaneous vasodilations\textsuperscript{117, 118} or vasoconstrictions\textsuperscript{119} in response to pharmacological stimuli. \textbf{The pharmacological properties of fluconazole and aspirin were used in Aim 3 to investigate the role of EETs and PGs in luminance flicker-induced retinal vasodilation.}

2.5.3 Vasoactive metabolites

For many years, the dominant hypothesis of how neuronal activity induces local hyperaemia centred on the release of vasoactive metabolites from neurons as part of a feedback process. Activated neurons would consume ATP at a faster rate and induce a local hypoxia as neurons consumed more oxygen to replenish their stores of ATP. This would lead to a build-up and release of vasodilatory metabolites, including adenosine and lactate. Adenosine is released by the consumption of ATP and adenosine inhibitors reduce activity-induced hyperaemia in the brain\textsuperscript{120}. Lactate, a by-product of glycolysis, is produced in the retina during flicker light stimulation\textsuperscript{121}. However, neither metabolite has been shown to directly mediate neuronal activity-induced hyperaemia in the retina.

Based on current evidence, vasoactive by-products of metabolism are probably not primary mediators of retinal hyperaemia during flicker stimulation. While metabolites like adenosine and lactate do induce vasodilation, the onset of hyperaemic responses within seconds of flicker stimulation suggests that neurons in the retina pro-actively increase their own blood supply\textsuperscript{88, 92}. In addition, there is now good evidence from pharmacological studies that neuronal activity is directly linked to the release of vasodilator signalling molecules, from either neurons themselves or other retinal support cells.

2.6 Diabetes

Diabetes mellitus (diabetes) is a term given to a group of chronic metabolic diseases characterised by hyperglycaemia\textsuperscript{43}. Globally, approximately 382 million people, 8.3\% of the world’s population, are estimated to have diabetes\textsuperscript{122}. This figure is expected to increase by 55\% to 592 million by 2035, with the greatest growth in low and middle-income countries. Type 1 diabetes accounts for 5-10\% of cases, while type 2 diabetes accounts for almost all of the remaining cases. Disturbingly, an estimated 46\% of people with type 2 diabetes are unaware of their ailment. The complications of diabetes are responsible for roughly 1.3\% of total disability-adjusted life years worldwide\textsuperscript{123}. This proportion is expected to increase as the prevalence of diabetes increases over the next 20 years.
At the fundamental level, diabetes arises due to insulin deficiency, insulin resistance or a combination of the two. Several causes of diabetes are recognised, however the vast majority of cases in practice can be classified as either type 1 or type 2. Type 1 diabetes is characterised by the destruction of insulin-producing pancreatic islet β-cells, generally by an autoimmune process. People with this type of diabetes invariably require insulin treatment to survive. Type 2 diabetes is characterised by insulin resistance and a relative deficiency of insulin. The mechanism of insulin resistance is not well understood, but a strong link with obesity is recognised. People with type 2 diabetes generally do not require insulin treatment until late in the disease.

Treatments aimed at replacing insulin or reducing insulin resistance have allowed people with diabetes to live long-term with the disease. However, chronic hyperglycaemia causes an insidious progression of damage and dysfunction in almost every organ in the body. The eyes are particularly susceptible to damage in diabetes, and people may experience vision loss or blindness if care is not taken.

2.7 Diabetic retinopathy

DR is a common and potentially blinding complication of diabetes. It is loosely defined as “the presence of typical microvascular signs in a person with diabetes”. Roughly one third of people with diabetes have clinically detectable DR lesions, such as microaneurysms or retinal haemorrhages. This translates to 126 million people worldwide. One in ten people with diabetes have vision-threatening DR, defined as proliferative DR (PDR) or diabetic macular oedema (DME) near the fovea. Improved glycaemic control appears to have reduced the incidence of DR in recent years. However, DR remains a significant cause of disability and is recognised as the most common cause of new blindness in working-age adults in the United States.

2.7.1 Pathogenesis

The DCCT and UKPDS clinical trials in patients with type 1 and type 2 diabetes, respectively, clearly established a strong link between long-term glycaemic control and the risk of DR. Chronic hyperglycaemia is thought to cause diabetic complications in part via the generation of mitochondrial oxidative stress. Oxidative stress may also result from the metabolism of fatty acids, which are increased with insulin resistance such as in type 2 diabetes. This may lead to the activation of several damaging biochemical pathways, including the polyol pathway, advanced glycation
end-products (AGEs), protein kinase C (PKC) and the hexosamine pathway. These biochemical pathways promote a chronic inflammatory response in the retina that eventually leads to capillary dropout and non-perfusion, retinal ischaemia and hypoxia-induced neovascularisation and vascular leakage. Although DR is classically considered to be a vascular complication of diabetes, neuronal and glial degeneration also occurs in the retina in people with diabetes. It is unclear whether luminance flicker-induced retinal vasodilation is reduced in diabetes because of retinal cell degeneration, acute functional impairments, or a combination of these two factors. To address this gap, Aim 4 investigated the impact of hyperglycaemia and oxidative stress on retinal flicker responses and neuronal function in people with type 1 diabetes.

Polyol pathway

The first step of the polyol pathway involves the reduction of glucose to sorbitol by aldose reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Sorbitol is then oxidised to fructose by sorbitol dehydrogenase and nicotinamide adenine dinucleotide (NAD$^+$). The consumption of NADPH is thought to render cells more vulnerable to oxidative stress. Aldose reductase is found in several retinal cells including pericytes, endothelial cells, Müller cells, ganglion cells and RPE cells. Furthermore, polymorphisms in the aldose reductase gene are associated with increased DR risk in people with type 1 and type 2 diabetes.

Aldose reductase inhibitors were evaluated in several clinical trials of DR. The Sorbinil Retinopathy Trial, the largest, found no significant benefit on DR progression in people with type 1 diabetes after 3-4 years of treatment. Sorbinil was also associated with serious adverse drug reactions in 7% of participants. Other trials of aldose reductase inhibitors found similar negative results. The disappointing results of these trials led to a loss of interest in these inhibitors for DR.

Advanced glycation end-products

AGEs are a group of long-lived compounds formed from reactions between reactive sugars and proteins. AGEs cause damage by three main mechanisms: 1) AGE-modified proteins have impaired functions; 2) AGEs in extracellular matrix impair communications with other matrix and cell components; and 3) binding with cell surface receptors for AGE promotes the transcription of pro-inflammatory genes.

A link between AGEs and DR was suggested by the finding of high vitreous levels of AGEs in people with PDR. In addition, AGEs added to cell cultures induces retinal pericyte apoptosis and Müller cell vascular endothelial growth factor (VEGF).
production\textsuperscript{140, 141}. AGEs in skin collagen biopsies from participants in the DCCT were found to be better 10-year predictors of DR than mean haemoglobin A1c (HbA1c) levels\textsuperscript{142}. Some skin AGEs can be measured non-invasively by autofluorescence. Skin autofluorescence was associated with DR in people with type 1 diabetes followed in the Epidemiology of Diabetes Interventions and Complications (EDIC) study\textsuperscript{143}. However, this association was not statistically significant when adjusted for mean HbA1c levels.

AGEs in plasma have limited value in the prediction of long-term diabetic complications due to their rapid clearance by the kidneys.

One inhibitor of AGEs, aminoguanidine, was evaluated in a clinical trial of 690 people with type 1 diabetes\textsuperscript{144}. Aminoguanidine had no effect on the primary outcome of serum creatinine doubling time, but reduced DR progression by 38% over three years. Despite these results, further evaluation was halted due to the development of serious renal complications in three people. Other treatments against AGEs are in development, but none have demonstrated any clear clinical benefit on diabetic complications\textsuperscript{145}.

\textit{Protein kinase C}

Several forms of PKC appear to be involved in the development of diabetic complications, especially the -\(\beta\) and -\(\delta\) forms\textsuperscript{146}. PKC-\(\delta\) decreases platelet-derived growth factor survival signalling and induces pericyte apoptosis\textsuperscript{147}. In contrast, PKC-\(\beta\) appears to promote pathological blood vessel proliferation. Vessel proliferation in oxygen-induced retinopathy was increased in transgenic mice overexpressing PKC-\(\beta2\) and decreased in PKC-\(\beta\) null mice\textsuperscript{148}. In bovine retinal endothelial cells cultured with VEGF, cell growth was enhanced by overexpression of PKC-\(\beta1\) or -\(\beta2\) and decreased by inactivation of the PKC-\(\beta2\) gene\textsuperscript{148}. Furthermore, VEGF-induced permeability was significantly reduced by PKC inhibitors in cultured bovine retinal endothelial cells\textsuperscript{149}.

One PKC-\(\beta\) inhibitor, ruboxistaurin (RBX), has been evaluated in human trials\textsuperscript{150-154}. Oral RBX, up to 16 mg twice daily for 28 days, improved retinal circulation time and retinal blood flow in 29 people with type 1 or type 2 diabetes and minimal DR\textsuperscript{150}. The PKC-\(\beta\) Inhibitor-Diabetic Retinopathy Study 2 evaluated the effects of oral RBX 32 mg once daily on DR end-points in 685 people with diabetes and moderate to severe non-proliferative DR (NPDR)\textsuperscript{151}. The study found that RBX reduced sustained moderate visual loss, first laser treatment for DME and the progression of DME to centre-involving stage over 36 months. It did not reduce the progression of DR, which
suggests that its benefit may be limited to DME. Furthermore, a 2-year open-label extension of this clinical trial found that participants initially randomised to RBX had significantly less sustained moderate visual loss than those randomised to placebo\textsuperscript{154}. Despite promising results for people with DME, RBX has not yet received pharmaceutical regulatory approval.

*Hexosamine pathway*

The hexosamine pathway generates uridine diphosphate-$N$-acetylglucosamine (UDP-GlcNAc)\textsuperscript{155}. This molecule is involved in the posttranslational modification of several proteins. For example, acylation by UDP-GlcNAc could promote endothelial dysfunction in people with diabetes\textsuperscript{156}. The hexosamine pathway has been linked to insulin resistance in type 2 diabetes\textsuperscript{157}. However, importance of this pathway in the development of DR remains relatively unexplored.

*Cellular changes*

Pericytes are vascular cells with contractile process that extend around the outside of blood vessels in the retina\textsuperscript{158}. The loss of these cells from retinal capillaries is one of the earliest features of DR\textsuperscript{159, 160}. Furthermore, areas of pericyte loss are associated with the development of microaneurysms, one of the first clinical signs of DR. The cause of pericyte loss in diabetes is not well understood. However, the apoptosis of cultured bovine pericytes induced by AGEs and PKC-$\delta$ suggests that biochemical mechanisms are involved\textsuperscript{141, 147}. The resolution of microaneurysms is accompanied by a loss of endothelial cells and non-perfusion of capillaries\textsuperscript{159}. In the late stages of DR, retinal neovascularisation occurs in an attempt to re-perfuse areas of retinal ischaemia. Retinal neovascularisation is strongly linked to increased VEGF production in people with PDR\textsuperscript{161}.

Retinal neurodegeneration is now recognised as an important feature of DR. Streptozotocin (STZ)-induced diabetic rats develop thinning of the inner plexiform and inner nuclear layers\textsuperscript{162}. Furthermore, retinal neural apoptosis is increased in both diabetic rodents and diabetic post-mortem human eyes. Inner retinal thinning was later confirmed non-invasively by optical coherence tomography (OCT) in diabetic humans with no or minimal DR\textsuperscript{163, 164}. Human and rodent studies suggest that retinal ganglion cells are the neurons most susceptible to damage in diabetes\textsuperscript{162, 165, 166}.

The mammalian retina contains two types of macroglial cells: Müller cells and astrocytes\textsuperscript{167}. Müller cells extend throughout the layers of the neurosensory retina,
whereas astrocytes are restricted to the inner retina. These macroglial cells are important for the integrity of the blood-retinal barrier\textsuperscript{168}. Astrocyte dysfunction and Müller cell gliosis occur early in diabetic rodent models and are accompanied by vascular leakage\textsuperscript{169, 170}. Reactive glial cells may contribute to retinal damage via the release of inflammatory molecules and impaired neuronal support\textsuperscript{171}. For example, glutamate metabolism by glial cells is impaired in STZ diabetic rats, which may lead to glutamate toxicity and neurodegeneration\textsuperscript{172}. Both AGEs and hypoxia promote Müller cell VEGF production\textsuperscript{140, 173}. These signals may contribute to increased Müller cell VEGF expression in diabetic human eyes\textsuperscript{174}.

\textbf{Figure 6.} ETDRS seven standard photographic fields for grading DR severity\textsuperscript{175}. 
Table 2. ETDRS DR severity scale\textsuperscript{176}.

<table>
<thead>
<tr>
<th>DR severity</th>
<th>Definition</th>
<th>One-year progression to high-risk PDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR absent (level 10)</td>
<td>No abnormalities</td>
<td>Not reported</td>
</tr>
<tr>
<td>Minimal NPDR (level 20)</td>
<td>MA only</td>
<td>Not reported</td>
</tr>
<tr>
<td>Mild NPDR (level 35)</td>
<td>MA plus H, HE, or SE</td>
<td>1.2%</td>
</tr>
<tr>
<td>Moderate NPDR (levels 43, 47)</td>
<td>H/MA = S/1-3, IRMA = D/1-5, or VB = D/1</td>
<td>3.6, 8.1%</td>
</tr>
<tr>
<td>Severe NPDR (level 53)</td>
<td>$\geq$2 of the above, H/MA $\geq$ S/4-5, IRMA $\geq$ M/1, or VB $\geq$ D/2-3</td>
<td>17.1%</td>
</tr>
<tr>
<td>Early PDR (levels 61, 65)</td>
<td>NVE $\geq$ D or NVD = D with VH and PRH = A or Q, or VH or PRH = D with NVE $&lt; M/1$ and NVD = A</td>
<td>33.4%</td>
</tr>
<tr>
<td>High-risk PDR (level 71)</td>
<td>VH or PRH $\geq$ M/1, NVE $\geq$ M/1 with VH or PRH $\geq$ D/1, NVD = D with VH or PRH $\geq$ D/1, or NVD $\geq$ M</td>
<td>N/A</td>
</tr>
</tbody>
</table>

MA = microaneurysms, H = haemorrhages, HE = hard exudates, SE = soft exudates, IRMA = intra-retinal microvascular abnormalities, VB = venous beading, NVE = new vessels elsewhere, NVD = new vessels disc, VH = vitreous haemorrhage, PRH = pre-retinal haemorrhage.

2.7.2 Risk factors

A longer duration of diabetes is a strong risk factor for DR. After 20 years of diabetes, almost all people with type 1 diabetes and 80% of people with type 2 diabetes will have visible signs of DR\textsuperscript{177, 178}. PDR and DME occur in 42% and 29% of people with type 1 diabetes after 25 years of disease, respectively\textsuperscript{179, 180}. However, it is important to note that these figures were obtained from studies conducted before the importance of tight glycaemic control was recognised. The incidence of DR and DME has improved in recent years\textsuperscript{181}.

Glycaemic control is the most important modifiable risk factor for DR. The DCCT\textsuperscript{20} and UKPDS\textsuperscript{21} found that intensive diabetes management, of which glucose control was a major feature, was strongly protective against DR. The DCCT found that for every 10% reduction in HbA1c there was a 39% reduction in the risk of DR progression\textsuperscript{182}. Hypertension is another important modifiable risk factor. Participants
with type 2 diabetes in the UKPDS with blood pressures greater than 140 mmHg were three times as likely to develop DR as those with blood pressures less than 125 mmHg. Current Australian guidelines recommend targets of ≤ 7.0% for the HbA1c and ≤ 130 mmHg for the systolic blood pressure in those with diabetes. Associations between lipids and DR have been inconsistent, although data suggest a link between lipids and hard exudates or DME. DR guidelines suggest targets of ≤ 2.5 mmol/l for low-density lipoprotein cholesterol and ≤ 2.0 mmol/l for triglycerides. **Aim 4a studied the specific effects of euglycaemia and hyperglycaemia on luminance flicker-induced retinal vasodilations, ganglion cell function and systemic arterial elasticity in people with type 1 diabetes.**

### 2.7.3 Grading of severity

The Early Treatment Diabetic Retinopathy Study (ETDRS) developed a classification system for the typical lesions of DR using fundus photographs. Consolidated information on the presence and severity of these lesions was then used to define several discrete levels of DR severity. Each increase in level on the DR severity scale was associated with approximately double the risk of progression to high risk PDR over one year (Table 2). High risk PDR was the main risk factor for visual loss or vitrectomy over five years, with an odds ratio of 13.7. The original ETDRS grading system was based on seven-field stereoscopic fundus photography of each eye with a 30° camera field of view (Figure 6). However, two-field fundus photography with newer 45° non-mydriatic cameras is now regarded as an acceptable alternative to the full seven fields. Formal grading of fundus photographs is the gold standard method for assessing DR severity. However, it is often impractical outside of research settings and does not convey information about retinal neuronal or vascular function. **Functional markers, such as luminance flicker-induced retinal vasodilation, may therefore provide useful additional information to help guide the timings of follow-up intervals and interventions in people with type 1 diabetes.**

### 2.7.4 Management

Basic management of DR involves good control of modifiable risk factors, particularly glycaemia, blood pressure, lipids and non-smoking. Good glycaemic control is particularly important to maintain over the long term. In an open label extension of the DCCT, the EDIC study, participants originally randomised to intensive diabetes management remained half as likely to have DR progression after four years relative to the conventional management group, despite equivalent HbA1c levels.
Similar results were reported for participants followed after the conclusion of the UKPDS\textsuperscript{188}. These observations led to the ‘metabolic memory’ hypothesis, whereby periods of poor glycaemic control may have persistent negative effects long after control is improved. The mechanism of this phenomenon is hypothesised to involve oxidative stress, AGEs and epigenetic histone modifications\textsuperscript{189}.

Specialist ophthalmic treatment is indicated in cases of PDR or vision-threatening DME. In PDR, panretinal laser photocoagulation is used to burn the peripheral retina and preserve central vision. The Diabetic Retinopathy Study found that panretinal photocoagulation for PDR reduced the risk of severe visual loss by upwards of 50\% over five years\textsuperscript{190}. The mechanism is thought to involve a decrease in hypoxia-induced VEGF production by peripheral retinal cells\textsuperscript{191}. Focal or grid laser is recommended for DME involving the centre of the macula. The ETDRS found that laser treatment for vision-threatening DME reduced the risk of moderate visual loss (doubling of the visual angle) by half\textsuperscript{192}. The side effects of laser treatment include impaired light-dark adaptation, reduced visual acuity and peripheral field loss\textsuperscript{126}. Recent clinical studies indicate that intravitreal injections of anti-VEGF agents or steroids may offer better visual outcomes for people with DME compared with laser alone\textsuperscript{193}. Steroids require fewer injections than anti-VEGF agents but are associated with accelerated cataract development and increased intraocular pressures. Steroids are therefore generally reserved for people with artificial intraocular lenses. Despite these treatments, a significant proportion of people with vision-threatening DR go on to develop visual loss. In people with type 2 diabetes, oral fenofibrate reduces the progression of existent DR and the need for laser treatment\textsuperscript{194, 195}. Fenofibrate has recently received regulatory approval for people with type 2 diabetes with existent DR and its clinical efficacy in people with type 1 diabetes is currently under investigation.

2.7.5 Follow-up

The purpose of follow-up in DR is to identify high-risk features early at a time where the risk of vision loss without treatment outweighs the risks of treatment\textsuperscript{22, 23}. People with type 1 diabetes should be examined within five years of their diagnosis for DR and every one to two years thereafter. Many people with type 2 diabetes already have signs of DR at the time of clinical diagnosis, which may be years after its onset, and should commence eye exams immediately. Signs of mild or moderate NPDR should prompt a referral to an ophthalmologist and should be followed up at least annually. At higher severity levels, follow-up should be performed approximately every
three months guided by the clinical scenario. Urgent referral to an ophthalmologist should be done for any person with diabetes who experiences sudden unexplained visual loss or who has any signs of foveal DME or neovascularisation.

2.8 Flicker light-induced retinal vasodilation in diabetes

Dynamic retinal imaging with flicker light stimulation has been proposed as a sensitive marker of retinal function. Its use in diabetes is particularly interesting, given the damaging effects of diabetes on the systemic microcirculation and the relative ease with which retinal blood vessels can be visualised. Cross-sectional studies have found that luminance flicker-induced retinal arteriole and venule dilations are reduced in people with type 1 and type 2 diabetes compared with non-diabetic controls. Furthermore, reduced flicker-induced dilations have been found in people with diabetes without DR. These findings suggest that retinal vasodilation during luminance flicker stimulation is impaired early in the course of diabetes. These responses may therefore represent sensitive markers of early retinal damage in diabetes that could be used to improve risk assessments for DR progression, guide follow-up intervals and act as surrogate end-points in clinical trials.

The mechanisms of luminance flicker-induced retinal vasodilation are incompletely understood, which makes it hard to interpret reduced responses in type 1 diabetes. Nitric oxide appears to be an important signalling molecule in these responses. However, diabetic people with impaired flicker responses were observed to have normal vascular responses to exogenous nitric oxide. This suggests that if nitric oxide signalling is impaired, it is at a level prior to its actions on vascular smooth muscle cells.

Pathological nitric oxide production by iNOS may lead to neurovascular dysfunction in diabetes. Upregulation of iNOS has been found in the retinas of diabetic rodents and humans. Furthermore, mice genetically deficient in iNOS are protected from retinal capillary degeneration, leukostasis and breakdown of the blood-retinal barrier after STZ-induced diabetes. Aminoguanidine is an iNOS inhibitor that attenuates increased levels of nitric oxide in the diabetic retina. It improved flicker light-induced retinal arteriole dilation in diabetic rodents. However, its other effects, including AGE inhibition, make it difficult to solely attribute this improvement to iNOS inhibition. Interestingly, insulin attenuates macrophage iNOS activity in diabetic rodents. Thus, insulin treatment in type 1 diabetes may reduce
pathological iNOS activation. The pathogenic role of nitric oxide in DR may be unique to iNOS, as deletion of the eNOS gene worsens the effects of experimental diabetes in mice\textsuperscript{205}.

Excessive levels of nitric oxide can react with superoxide to form peroxynitrite\textsuperscript{206}. In turn, peroxynitrite reacts with retinal proteins and promotes neurotoxicity\textsuperscript{207-211}. Müller cells appear to be an important source of nitric oxide in diabetes\textsuperscript{198, 212}. Diabetic Müller cells have impaired glutamate uptake\textsuperscript{213} and may contribute to increased glutamate levels in diabetes\textsuperscript{172}. Thus, diabetic Müller cells may promote nitric oxide toxicity directly via the induction of iNOS and indirectly via impaired glutamate uptake. Ganglion cells express NMDA receptors and would be particularly susceptible to glutamate toxicity\textsuperscript{96}. Thus, pathological nitric oxide and NMDA-mediated toxicity may contribute to retinal ganglion cell dysfunction and impaired neurovascular function in type 1 diabetes.

It is unclear if abnormalities in other signalling pathways, such as those involving arachidonic acid metabolites, may be involved in changes in diabetes. One study found a moderate correlation between the PERG transient response N95 implicit time and flicker-induced retinal arteriole dilation in people with diabetes without DR and non-diabetic controls\textsuperscript{18}. This implies a relationship between ganglion cell dysfunction and impaired flicker responses, which is consistent with laboratory observations of early ganglion cell loss in diabetes\textsuperscript{162, 165, 166}. However, another study of people with a shorter duration of type 1 diabetes found reduced flicker responses in the absence of impaired PERG responses\textsuperscript{19}. This suggests that vascular dysfunction may occur prior to neuronal dysfunction, although the latter may still contribute to impaired flicker responses after a longer duration of diabetes. \textbf{Aim 4a studied changes in luminance flicker-induced retinal vasodilation at different blood glucose levels in people with type 1 diabetes to better understand why responses become impaired in this population.}

\section*{2.9 Electroretinography in diabetes}

ERG findings support an element of dysfunction in most classes of retinal neurons in people with diabetes. The PERG and photopic negative response (PhNR) of the full-field ERG are consistently impaired in people with diabetes\textsuperscript{214-218}. These responses are thought to be specific for ganglion cells\textsuperscript{83, 85}. Furthermore, blue flash PhNR amplitudes were found to be reduced in adolescents with type 1 diabetes without
evident DR. This could reflect specific abnormalities in the small bistratified ganglion cells that carry the blue ON signal. Amplitudes and implicit times of the full-field ERG OPs are frequently impaired in diabetes and appear to be sensitive markers of neuronal dysfunction. These changes in the OPs probably reflect damage to the amacrine or bipolar cells. Bipolar cell damage is also suggested by impaired mfERG responses in people with DR. Interestingly, retinal areas with impaired mfERG responses were 21 times more likely than areas with normal responses to develop new retinopathy after one year in eyes with NPDR at baseline. Outer retinal dysfunction is not consistently found in diabetic ERG studies. However, impaired photoreceptor sensitivities and S-cone dysfunction have been reported. Together, these ERG findings indicate that neuronal dysfunction is an important feature of DR, particularly in the inner retina. These electrophysiological parameters may therefore represent additional markers of retinal damage to complement traditional DR risk assessments.

Aim 4 studied whether ganglion cell function, measured by the PERG, changes in response to different blood glucose levels or antioxidants in people with type 1 diabetes.

2.10 Systemic arterial elasticity in diabetes

Systemic arterial elasticity is a marker of interest in diabetes vascular research that can be measured non-invasively using arterial pulsewave tonometry. Cross-sectional data indicate that systemic arterial elasticity is reduced in people with type 1 diabetes compared with non-diabetic controls. Furthermore, acute hyperglycaemia rapidly increases arterial stiffness in both type 1 diabetes and non-diabetic human volunteers. No published study has demonstrated a link between systemic arterial elasticity and retinal vascular function. It is also not known whether retinal vascular function is rapidly impaired by hyperglycaemia in people with type 1 diabetes, as occurs in the systemic arteries. Aim 4a studied changes in systemic arterial elasticity during euglycaemia and hyperglycaemia in type 1 diabetes. In addition, Aim 4b studied whether antioxidants might affect these responses.

2.11 Hyperglycaemia and vascular function

It remains unclear whether impaired retinal vascular dysfunction occurs linearly in diabetes or whether it fluctuates with glucose control. Hyperglycaemia is directly linked to endothelial dysfunction and vascular stiffening and could similarly
interfere with retinal vasodilations. One study found smaller flicker-induced venule
dilations during hyperglycaemic clamps in non-diabetic people using somatostatin to
suppress endogenous insulin secretion\textsuperscript{231}. This link has not previously been investigated
in type 1 diabetes, where somatostatin would not be required.

Hyperglycaemic clamps have been used to directly investigate the effects of
hyperglycaemia on systemic arterial elasticity. Acute hyperglycaemia rapidly induced
arterial stiffening in people with and without type 1 diabetes, measured by radial artery
tonometry\textsuperscript{37, 38}. These studies similarly used somatostatin to suppress insulin secretion
in non-diabetic people. However, type 1 diabetic participants did not receive insulin as
part of the study protocol, and their baseline glucose levels were significantly higher
than non-diabetic participants. Insulin and glucose have vasodilatory actions\textsuperscript{232, 233}. However, insulin may reduce, rather than increase, retinal blood flow in people with
type 1 and type 2 diabetes\textsuperscript{234, 235}. I speculate that neuronal function in the retina would
be unlikely to fluctuate significantly with changing blood glucose and insulin levels,
although no study has investigated this possibility. \textbf{Aim 4a studied the effect of
euglycaemia and hyperglycaemia on retinal and systemic functional responses in
type 1 diabetes.}

\subsection*{2.12 Oxidative stress and antioxidants}

Hyperglycaemia has been directly linked to oxidative stress. Rat mesangial cells
cultured with high glucose developed glucose receptor-mediated oxidative stress within
15 minutes\textsuperscript{236}. Levels of plasma F2-isoprostanes and nitrosative stress were increased
following an oral glucose load in people with type 2 diabetes\textsuperscript{237, 238}. Increased levels of
nitrosative stress have also been found in healthy people within two hours of acute
hyperglycaemia\textsuperscript{239}. Other studies suggest that glucose variability has more of an impact
on oxidative stress than acute hyperglycaemia \textit{per se}. Urinary F2-isoprostane levels
were strongly correlated with the mean amplitude of glycaemic excursions over 24
hours in people with type 2 diabetes\textsuperscript{240}. In contrast, no correlation was found between
urinary F2-isoprostanes and mean glucose levels. Another study found that oscillating
glucose levels induced greater levels of oxidative stress than sustained hyperglycaemia
in non-diabetic and type 2 diabetic people\textsuperscript{241}.

Oxidative stress in diabetes has additionally been suggested from studies of
antioxidants. Levels of vitamin C are generally lower in people with diabetes\textsuperscript{242}. Furthermore, total antioxidant levels are decreased following an oral glucose load\textsuperscript{243}. 

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Vitamin C improves endothelial function in people with type 1 diabetes\textsuperscript{244} and protects against hyperglycaemia-induced vascular dysfunction\textsuperscript{37, 39, 40, 44}. Simultaneous control of glucose levels and oxidative stress with insulin infusions and vitamin C, respectively, normalised endothelial function in people with type 1 diabetes\textsuperscript{245}. This suggests that insulin and antioxidants may have complementary benefits on vascular functions in type 1 diabetes.

If diabetic complications arise in part due to hyperglycaemia-induced oxidative stress, then systemic antioxidants might provide some protection against DR and other neuronal and vascular complications. Unfortunately, specific clinical trials to evaluate the potential role of antioxidants in diabetic complications have thus far been limited\textsuperscript{246}. Most trials were designed to evaluate the effect of antioxidants on cardiovascular outcomes in high-risk patients and included patients with diabetes as a subset of the total sample. One study, the Steno-2 study, included low dose antioxidants as part of an intensive risk factor-lowering intervention in people with type 2 diabetes\textsuperscript{247}. Participants randomised to receive intensive treatment were half as likely as those in the standard care group to experience cardiovascular or microvascular events over a mean follow-up of 7.8 years. However, as antioxidant treatment was only a small part of a large treatment strategy it is difficult to draw conclusions on its effects. The AREDS found that oral antioxidants could delay the progression of certain forms of AMD\textsuperscript{27}. Similar benefits may be hypothesised in DR, given the hypothesised role of oxidative stress in this complication\textsuperscript{24}. Specific studies are therefore needed to study the potential benefits of antioxidants in DR.

Vitamin C is a natural antioxidant that at high doses can reduce acute hyperglycaemia-induced endothelial dysfunction\textsuperscript{39, 44} and arterial stiffness\textsuperscript{37} in non-diabetic people. Furthermore, vitamin C improves endothelium-dependent vasodilation in people with both major types of diabetes\textsuperscript{244, 245, 248} and blocks hyperglycaemic impairment of systemic endothelial function in type 1 diabetes\textsuperscript{40}. It is unknown whether vitamin C could improve vascular function in the retina in diabetes or protect against vascular dysfunction during periods of hyperglycaemia. Therefore, Aim 4b investigated whether antioxidant treatment with vitamin C could improve luminance flicker-induced retinal vasodilation, retinal ganglion cell function or systemic arterial elasticity in people with type 1 diabetes.
2.13 Context to the thesis

This chapter has presented a detailed discussion of the neuronal organisation of the retina and the physiological processes activated by different characteristics of light stimulation. This information is fundamental for a thorough understanding of the neuronal and vascular mediators of flicker light-induced retinal vasodilation. Furthermore, I have presented an overview of diabetes and its main ocular complication, DR. The biochemical and cellular changes that occur in the retina during diabetes are probable contributors to impaired flicker light-induced retinal vasodilation in people with diabetes. A major focus of this thesis was on the potential causes of short-term reductions in these responses, which at the time of writing have not yet been completely described.

Luminance flicker-induced retinal vasodilation is a relatively novel marker of retinal vascular function. Good reproducibility of functional markers is important when within-subject changes are under investigation. With 30 – 60 minutes between tests, flicker-induced arteriole and venule dilations have reported $R^2$ values of 0.85 – 0.94 and 0.19 – 0.80, respectively\textsuperscript{30, 31}. Aim 1 studied whether these correlations are maintained or whether follow-up responses are reduced with a very short rest period. \textbf{The results of Aim 1 are presented in Chapter 4.}

Larger retinal vasodilations during luminance flicker stimulation occur with a higher modulation depth\textsuperscript{90}. This reflects the contrast in retinal illumination between the ON and OFF flicker phases and probably involves opposing achromatic input from ON and OFF centre parasol ganglion cells\textsuperscript{60}. It is not clear whether ambient lighting conditions may affect the magnitude of luminance flicker-induced retinal vasodilations, as this may alter the effective modulation depth at the retina to a given flicker stimulus. This is important to know for studies where it is not feasible to conduct tests under reduced ambient lighting conditions. \textbf{Aim 2} studied whether higher ambient lighting affects these vascular responses. \textbf{The results of Aim 2 are presented in Chapter 5.}

Abnormalities in the signalling pathways that mediate flicker-induced retinal vasodilations may contribute to impaired responses in diabetes. Several lines of evidence point to nitric oxide as a principal mediator of flicker-induced retinal vasodilations\textsuperscript{5-7}. Dysfunctional nitric oxide signalling could therefore be a cause of impaired vascular responses in diabetes. However, observations from rodents have implicated EETs and PGs as important alternate signalling molecules\textsuperscript{32, 33}. \textbf{Aim 3} investigated the potential role of these molecules in healthy human participants. These
findings could suggest additional lines of inquiry in people with diabetes. **The results of Aim 3 are presented in Chapter 6.**

Haemodynamic parameters in the retinal and systemic circulations are known to fluctuate in people with diabetes in line with glucose levels and insulin administration. For example, retinal blood flow was increased in people with type 1 diabetes but reduced to levels comparable with non-diabetic people when glucose levels were fixed at a normal level during insulin infusions. In addition, systemic arterial stiffness increased rapidly in people with type 1 diabetes during hyperglycaemia. Luminance flicker-induced retinal vasodilation may similarly improve during insulin administration or worsen during hyperglycaemia. **Aim 4a** investigated this hypothesis in people with type 1 diabetes.

Hyperglycaemia is hypothesised to lead to diabetic complications, such as DR, in part via the generation of oxidative stress. If oxidative stress interferes with vascular function, then antioxidants might improve or protect vascular function in the retina. Vitamin C was shown to protect against hyperglycaemia-induced systemic arterial stiffening in people without diabetes. **Aim 4b** reported on whether similar effects may be observed on luminance flicker-induced retinal vasodilation in people with type 1 diabetes. **The results of Aims 4a and 4b are presented in Chapter 7.**

This thesis adds important knowledge of the mechanisms of luminance flicker-induced retinal vasodilation and the potential causes of impaired responses in people with type 1 diabetes. This may lead to the use of these flicker responses as surrogate markers of vascular function in clinical trials to develop better treatments for DR. The results of this doctoral project may also help develop new functional tests to improve clinical risk assessments for DR and reduce the incidence of blindness from this complication.
CHAPTER 3. METHODS
3.1 Introduction

This chapter describes the general methodology used throughout this doctoral project. I have organised the sections below into common themes and described specific differences in techniques between each aim where applicable. Much of this information is described again in Chapters 4 to 7, which are presented in paper style.

3.2 Ethical approval

Aims 1 to 3 of my doctoral project were conducted at the RVEEH. The studies to address these aims had ethical approval (project number 12/1094H) from the RVEEH Human Research and Ethics Committee (HREC). Aim 4 was conducted at the Endocrine Testing Area, St Vincent’s Hospital Melbourne. The study to address this aim had ethical approval (project number 096/13) from the St Vincent’s Hospital Melbourne HREC-D (drug, device, and non-standard clinical interventions). Aims 3 and 4 required the use of drugs for non-standard clinical indications. Thus, Clinical Trial Notification forms were submitted to the Therapeutic Goods Administration for these two aims. All research protocols followed the tenants of the Declaration of Helsinki. Written informed consent was obtained from all participants. I allowed all participants sufficient time to consider the consent form prior to signing and verbally explained the nature, risks and potential benefits of the research. I answered any questions my participants may have had.

3.3 Project funding

Funding was obtained for HREC project 12/1094H from the RVEEH under the Small Research Grants Scheme ($9,182). In addition, a Pilot and Feasibility Grant was obtained from the JDRF Australia to investigate Aim 4 ($78,584). These funds covered the costs of personnel required for the projects, participant reimbursements, administrative fees, medical supplies and laboratory tests.

3.4 Recruitment

A total of 72 adults (60 non-diabetic and 12 type 1) participated in this doctoral project. Twenty healthy adults were recruited from the CERA staff and their associates each for Aims 1 and 2. A further 20 healthy adults were recruited from the University of Melbourne student population and the CERA for Aim 3. Twelve otherwise healthy adults with type 1 diabetes were recruited for Aim 4 from the University of Melbourne
student population and through notices with Diabetes Australia (Victoria) and the JDRF (Australia).

Participants were eligible for Aims 1 to 3 if they had no self-reported chronic medical disease, no significant eye problems other than a need for corrective lenses, no history of epilepsy, no known allergy to the study drugs, were non-smokers and were not pregnant or lactating. Participants were eligible for Aim 4 if they had a self-reported history of type 1 diabetes with insulin treatment, no known diabetic complications at the time of recruitment and otherwise fulfilled the criteria in Aims 1 to 3 described above. Myopia was not a formal exclusion criterion, however one volunteer in Aim 2 with high myopia (spherical equivalent -6.75) could not be included because I could not identify blood vessels near the optic disc sufficiently wide enough for the optical resolution of the imaging equipment. Participants in Aim 3 were also screened for: 1) undiagnosed retinal pathology on 45° macula and optic disc-centred fundus photographs with digital non-mydriatic camera (CR-2, Canon, Melville, NY); 2) cardiac conduction abnormalities on a 12-lead electrocardiogram (ELI 150 Rx, Mortara Instrument, Milwaukee, WI); and 3) any clinical abnormalities found during a general physical examination. However, no participant in Aim 3 was excluded based on these additional screening tests.

Healthy participants were offered $20 per visit as compensation for their time and travel costs. Participants with type 1 diabetes were offered $50 per visit in recognition of the greater time commitment required.

3.5 Study designs
3.5.1 Study 1 (Aim 1)

I conducted a repeated measures observational study of luminance flicker-induced retinal vasodilation in the right eyes of 20 healthy volunteers. All participants had their age, sex, best-corrected visual acuity (BCVA), refractive error (spherical equivalent), intraocular pressure (IOP), blood pressure, heart rate and body mass index (BMI) recorded at the beginning of the study. Abstention from alcohol and caffeine-containing products was requested from all participants in the 12-hour period prior to the study.

Luminance flicker-induced retinal arteriole and venule dilations were measured three times under reduced ambient lighting. After the initial test, the test was repeated with five minutes rest between flicker periods of the first and second test. A third test
was performed with 30 minutes between flicker periods of the second and third test. Participants were seated in a quiet area with normal room lighting between the second and third tests.

3.5.2 Study 2 (Aim 2)

I conducted a balanced crossover study of luminance flicker-induced retinal vasodilation under reduced and normal ambient lighting conditions in the right eyes of 20 healthy volunteers. The same baseline participant characteristics were recorded as for Study 1. Similarly, abstention from alcohol and caffeine-containing products was requested in the 12 hours prior to the study.

Luminance flicker-induced retinal vasodilation was measured twice with at least 20 minutes between consecutive periods. From Study 1, I found that sensitivity of the retinal arteriole response to flicker light stimulation recovers within 30 minutes. However, given that dark adaptation in cones after exposure to bright light is complete within five to 10 minutes, I chose a rest period of 20 minutes for Study 2 to improve convenience for my participants. Ten participants were tested under reduced followed by normal ambient lighting (sequence 1), while 10 participants were tested under normal followed by reduced ambient lighting (sequence 2). Normal ambient lighting was delivered by three sets of two ceiling-mounted 1.2 meter-long 36 watt fluorescent lights positioned approximately two meters above the imaging equipment. Reduced ambient lighting was achieved by turning off these lights.

3.5.3 Study 3 (Aim 3)

Crossover study

I conducted a balanced crossover study of luminance flicker-induced retinal vasodilation in the right eyes of 12 healthy volunteers. This study had three treatment conditions: 1) no drug; 2) oral fluconazole 400 mg (Sandoz, Pyrmont, Australia); and 3) dispersible aspirin 600 mg (Reckitt Benckiser, West Ryde, Australia). I used fluconazole and aspirin as inhibitors of EETs and PGs, respectively, to study the role of EETs and PGs in luminance flicker-induced retinal vasodilation. Participants were tested under each treatment condition across three visits, each separated by at least two weeks. Two participants were assigned to each of the six possible orders of drug treatments. The same baseline characteristics were recorded as for Studies 1 and 2, except that BCVA and refractive error were not recorded. BCVA and refractive error were not included in the original study design, but these parameters were not necessary
for my main outcomes. Abstention from alcohol and caffeine-containing products was requested in the 12 hours prior to each visit.

At each visit, participants had a 20-gauge intravenous cannula inserted in an antecubital fossa vein for blood sampling. Participants were seated in a quiet area for 30 minutes after cannulation. Luminance flicker-induced retinal vasodilation was measured under reduced ambient lighting at each visit and one hour after drug ingestions to allow sufficient time for absorption. Venous blood samples were collected in 4-ml ethylenediaminetetraacetic acid (EDTA) tubes before and after imaging tests. Blood samples were immediately centrifuged at 2,200 g and 4°C for 10 minutes. Plasma samples were then transferred to microcentrifuge tubes in 500 µl aliquots and stored at -80°C. Frozen samples were transferred to the NHMRC Clinical Trials Centre for prostaglandin E₂ metabolite assays. Separate samples were transferred to the University of Melbourne Department of Metabolomics for quantification of fluconazole and salicylic acid levels.

Repeated measures study

I measured luminance flicker-induced retinal vasodilation before and after ingestions of oral fluconazole 400 mg or dispersible aspirin 600 mg using the right eyes of 6 participants per drug. I obtained measurements at 30-minute intervals for two hours after drug ingestions. Measured participant characteristics were identical to the crossover study described above. However, unlike the crossover study, repeated measures study participants were not cannulated and blood samples were not obtained.

3.5.4 Study 4 (Aims 4a and 4b)

For Study 4, I studied participants with type 1 diabetes. The same participant characteristics were recorded as for Studies 1 and 2. I asked participants to cease any vitamin and herbal supplements from two weeks before their first visit and until after their second visit. Participants were requested to fast from midnight the night before each visit and were given individualised instructions for managing their insulin doses.

Participants were seen at two visits separated by at least two weeks. Systemic arterial elasticity, PERG transient and steady-state responses and luminance flicker-induced retinal vasodilation were measured, in that order, three times per visit: 1) at baseline after an overnight fast; 2) after one hour of insulin infusion (6 pmol/kg/min.) and 30 minutes of euglycaemia (6 mmol/l); and 3) after 30 minutes of hyperglycaemia (15 mmol/l). Tests were conducted under normal ambient lighting for safety reasons (510 lux at the eye). Insulin and glucose infusions continued throughout euglycaemic
and hyperglycaemic tests. Topical tropicamide 1% (Bausch & Lomb, Macquarie Park, Australia) was instilled in the right eye prior to the first and second series of functional tests to permit the assessment of luminance flicker-induced retinal vasodilation. Vitamin C (2 g in 100 ml sodium chloride 0.9%; Biological Therapies, Braeside, Australia) was given at one visit and placebo (100 ml sodium chloride 0.9%) was given at the other visit immediately prior to the initiation of hyperglycaemia. Both investigators and participants were masked to these treatments. Baseline urine and blood samples were collected prior to insulin infusions and sent to St. Vincent’s Pathology (Melbourne, Australia) to test for HbA1c, serum creatinine and the urine albumin:creatinine ratio.

**Glucose clamp**

I used a modified two-stage euglycaemic and hyperglycaemic clamp based on the technique of DeFronzo and colleagues (Figure 7)\textsuperscript{250}. After baseline tests, one 20 gauge intravenous cannula was inserted into an antecubital vein of each arm. One cannula was used for drug infusions and the other was for blood samples. Insulin (Actrapid, Novo Nordisk, Baulkham Hills, Australia) was infused at a constant rate of 6 pmol/kg/min. Glucose 25% was infused at a rate necessary for the euglycaemic glucose level (6 ± 1 mmol/l). An iterative computer program was used to guide glucose infusion rates\textsuperscript{251}. Whole blood glucose levels were measured every 10 minutes with the YSI STAT Plus (YSI Incorporated, Yellow Springs, USA) and the glucose infusion rate was adjusted accordingly. If the initial glucose level was ≥ 10 mmol/l before the start of the insulin infusion, an individualised correction dose was given over 10 minutes to reduce the glucose level to 6 mmol/l within 30 minutes. Functional tests were repeated after a minimum of one hour of insulin infusion and 30 minutes of euglycaemia. Vitamin C or placebo was then infused over 10 minutes and the glucose infusion rate was increased to raise the glucose level to 15 mmol/l over approximately 30 minutes. After hyperglycaemia was achieved, the infusion rate was reduced to maintain glucose levels in the hyperglycaemic range (15 ± 1 mmol/l). Functional tests were repeated a third time after 30 minutes of hyperglycaemia. Insulin and glucose infusions were then ceased. Participants were provided with a meal at the end of each visit and were observed for 45 minutes.
3.6 Haemodynamic measurements

Systolic and diastolic blood pressures were measured with non-invasive blood pressure cuffs positioned on the arm over the brachial artery. Blood pressure was recorded in a seated position, except in Study 4 where participants were supine. Blood pressure was recorded manually in Study 1 with a manual sphygmomanometer (Tycos, Welch Allyn, USA) and stethoscope (Littmann Master Cardiology, 3M, USA). Heart rate was calculated from palpation of the radial artery for 15 seconds. Blood pressures and heart rate were recorded automatically in Studies 2 and 3 with an automatic upper arm sphygmomanometer (HEM-7000-C1L, Omron Healthcare, Lake Forest, IL). Blood pressures and heart rate were recorded automatically in Study 4 once using the HD/Pulsewave CR-2000 system (Hypertension Diagnostics, Missesota, USA).

3.7 Intraocular pressure

IOP was measured after topical instillation of oxybuprocaine hydrochloride, 0.4%, and fluorescein sodium with a slitlamp-mounted Goldmann applanation
tonometer (Haag-Streit, Bern, Switzerland). IOP was measured in the same way throughout my doctoral project.

3.8 Best corrected visual acuity

BCVA was measured with a logarithm of the minimum angle of resolution (LogMAR) chart at a distance of four metres. Participants were required to correctly identify three out of five letters on each line to be awarded the LogMAR score for that line. Participants unable to read the LogMAR 0.00 line were refracted with the assistance of a trained orthoptist and their spherical equivalent refractive error was recorded. Participants able to read to the LogMAR 0.00 line or below without correction were assumed to have a refractive error of 0.00.

3.9 Ambient illuminance

Illuminance was measured in Studies 2 and 4 with an EasyView Digital Light Meter (EA30, Extech Instruments, USA). Measurements were taken at the level of the eye to estimate illuminance at the retina. I estimated the effective modulation depth of flicker in Study 2 according to the Michelson contrast:

\[ D = \frac{I_1 - I_2}{I_1 + I_2} \]

where \( D \) is the modulation depth, \( I_1 \) is the ON flicker illuminance and \( I_2 \) is the OFF flicker illuminance. Luminance is measured in Lux; the modulation depth is a ratio and has no units.

3.10 Luminance flicker-induced retinal vasodilation

Retinal arteriole and venule dilation during luminance flicker stimulation was measured in the right eye after dilation with tropicamide 1% (Bausch & Lomb, Macquarie Park, Australia) with the DVA (IMEDOS, Jena, Germany). The system uses green light illumination (530-600 nm) to provide the greatest contrast between retinal blood vessels and the surrounding tissue. Erythrocytes within the vessels have a maximum absorbance of between 400-620 nm, whereas most surrounding tissue reflects light in this range\(^{252}\). The reflected light is detected by a charge-coupled device (CCD) camera and used to estimate the vessel diameters. Diffuse luminance flicker is delivered by the DVA system over the entire visual field by way of an optoelectronic shutter that
interrupts the light source with a bright-to-dark ratio of 25:1 at a frequency of 12.5 Hz. This frequency lies near to values previously shown to maximise retinal vasodilation and blood flow during flicker stimulation\textsuperscript{11,\textit{90},\textit{91}}.

Participants were seated and instructed to fixate on the tip of a fixation bar within the Zeiss FF450plus mydriatic camera (Carl Zeiss AG, Jena, Germany) while the fundus was examined with a viewing angle of 30°. The average luminance of the light source was maintained at 130 cd/m\textsuperscript{2}, measured with an ILT1700 Research Radiometer (International Light Technologies, Peabody, USA). A unique, high-contrast region of the fundus was selected as a target for the eye-tracking module of the DVA software. I then selected a straight segment of an arteriole and venule located approximately one half to two disc diameters from the optic disc margin. I preferred segments located in the superior temporal retina and at least one diameter from any bifurcation or neighbouring vessels. The same vessel segments were used for repeated tests.

Vessel diameters were recorded by the DVA software in MU, where 1 MU is approximately equal to 1 \( \mu \text{m} \)\textsuperscript{28}. Diameters were measured at 1 MU intervals along the length of each segment at 40-millisecond intervals, corresponding to a sample rate of 25 Hz. Each test took 350 seconds to complete. This consisted of 50 seconds of constant light, followed by three cycles of 20 seconds of diffuse luminance flicker at 12.5 Hz and 80 seconds of constant light.

Baseline arteriole and venule diameters (MU) were defined as the mean vessel diameter in the 30 seconds immediately prior to flicker. Maximum dilation (%) was defined as the increase in diameter relative to baseline after 20 seconds of flicker (\textbf{Study 1}) or during 20 seconds of flicker (\textbf{Studies 2 to 4}). The time of maximum dilation (sec.) was recorded for Study 2. However, this marker was not recorded for subsequent studies after it became clear that vessels were generally still dilating at the end of the 20-second flicker period. The AUC during flicker (\% x sec.) was calculated for \textbf{Studies 2 and 4} in STATA version 12.1 (StataCorp LP, College Station, TX).

The in-built DVA analysis software was used for \textbf{Studies 1 to 3}. In \textbf{Study 4}, vessel diameter-time data were exported to STATA to provide greater flexibility with data analyses. Three periods of 100 seconds were defined per test. Each period consisted of the 30 seconds pre-flicker, 20 seconds of flicker and 50 seconds post-flicker. The periods were superimposed and locally weighted scatterplot smoothing
(LOWESS) with a bandwidth of 2% was applied to smooth vessel diameter-time data. Linear interpolation was used for sample intervals with missing data.

### 3.11 Pattern electroretinogram

PERG studies were performed in **Study 4**. PERG studies were conducted on the left eye only according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standard\(^8\). Participants wore their own corrective lenses if required. Retinal potentials were recorded with DTL fibre electrodes positioned along the lower eyelid margin. Gold cup skin electrodes were positioned over the lateral canthus and central forehead as reference and ground electrodes, respectively. Electrode impedances were tested and were deemed acceptable if less than 5 kΩ. Signals were amplified by the Espion E\(^3\) Electroretinography System (Diagnosys LLC, Lowell, USA) with a sampling rate of 1.2 kHz and band-pass filter of 0.625 to 100 Hz at a digital resolution of 32 bits. Potentials greater than ±50 mV were rejected as artefacts.

Participants were seated 57 cm from a 21-inch Cornerstone p1650 cathode ray tube monitor (Cornerstone, Fremont, USA). The monitor was connected to a Pattern Stimulus Generator (Diagnosys LLC, Lowell, USA) with a frame rate of 100 Hz. Stimuli were stationary black and white checkerboards with a mean luminance of 54 cd/m\(^2\) and contrast of 100%. Each PERG study had three steps. In step one, the transient response was measured from the mean of 150 sweeps of 200 milliseconds with check sizes of 0.8° and a reversal rate of 1 Hz. I increased the reversal rate to 8.33 Hz in step two to measure the steady-state response. I then increased the check sizes to 7° in step three. For steps two and three I recorded the mean of 50 sweeps of one second.

I measured the amplitudes and implicit times of the N35, P50 and N95 components of the transient response. The positions of these three components were taken from where they would appear with an idealised response (Figure 3). N35 amplitudes were measured as the potential relative to the start of the sample recording. P50 amplitudes were measured as the potential relative to the N35 amplitude; N95 amplitudes were measured as the potential relative to the P50 component. Implicit times of the N35, P50 and N95 components were taken as the absolute time elapsed from the start of the sample recording.

I assessed the PERG steady-state response using frequency domain analyses from eight cycles of 120 milliseconds. The signal-averaged data were exported to STATA version 12.1 and the mean was subtracted from the signal. The discrete Fourier
transform was then used to obtain the amplitudes of the frequency components. The second harmonic frequency (i.e. 16.67 Hz; 2F) was the component of interest. The proportion of the steady-state response represented by the 2F and the ratio of the amplitudes with 0.8° and 7° check sizes was also extracted.

3.12 Radial artery pulsewave tonometry

Radial artery pulsewave tonometry was performed in Study 4 with the HD/Pulsewave CR-2000 (Hypertension Diagnostics, Minnesota, USA). Participants were positioned in a supine position for five minutes prior to measurements. An appropriately sized non-invasive blood pressure cuff was applied to the left arm while the right wrist was splinted to reduce movement and stabilise the radial artery. A pressure tonometer was positioned over the right radial artery and adjusted to the point of highest signal strength relative to the blood pressure. Arterial waveforms were recorded from one period of 30 seconds and the diastolic portion was digitised at a sample rate of 200 Hz. Pressure waveforms were automatically fitted to a modified Windkessel model to determine the systemic vascular resistance and the large (C1) and small (C2) artery elasticity as previously described\textsuperscript{253}. The system automatically calculated the systemic vascular resistance (SVR; mmHg x min/l), LAE (ml/mmHg x 10) and SAE (ml/mmHg x 100).

3.13 Retinal photography

Two-field colour fundus photography was done for study eyes in Studies 3 and 4. Images were centred on the macula and optic disc and taken with a Canon CR-2 digital non-mydriatic camera (Canon, Melville, NY) with a viewing angle of 45°.

Participants in Study 4 had type 1 diabetes. Therefore, DR was assessed by independent graders at the CERA according to a modification of the ETDRS severity scale\textsuperscript{176}.

3.14 Statistical analysis

All statistical analysis was conducted in STATA version 12.1 (StataCorp LP, College Station, TX). Descriptive statistics for participant characteristics were reported as means (SD) (Studies 1, 2 and 3) or medians with IQR (Study 4). Comparisons of participant characteristics in Study 3 were performed by ANOVA for continuous variables or Fisher’s exact test for categorical variables. All group means were assessed
by ANOVA with post-hoc pairwise comparisons for significant differences. Bonferroni’s (Study 1), Dunnett’s (Study 3) or Sidak’s adjustments (Study 4) were used to correct for multiple post-hoc comparisons as appropriate. In Study 4, within-subjects Pearson’s correlation coefficients were reported for DVA parameters (arteriole and venule maximum dilations and AUC during flicker) with glucose and insulin levels, haemodynamic parameters, ganglion cell response amplitudes (PERG P50, N95 and 2F amplitudes) and systemic arterial elasticity parameters. Within-subjects Pearson’s correlation coefficients were calculated using the methods described by Bland and Altman\textsuperscript{254}. Two-tailed $P < 0.05$ was considered significant for all statistical tests.
CHAPTER 4. RETINAL ARTERIOLAR DILATION TO FLICKER LIGHT IS REDUCED ON SHORT-TERM RE-TESTING
4.1 Context to the paper

This paper is presented *in lieu* of a traditional thesis chapter. It addresses *Aim 1* of my doctoral project, “To investigate the effect of repeated testing on luminance flicker-induced retinal vasodilations in healthy people”. It has been lightly edited from its published version for Australian spelling, presentation and consistency within this doctoral thesis. The citation is:


The nature and proportion of my contributions and those of my collaborators to the work presented in this chapter are described in the Preface of this thesis.
RETINAL ARTERIOLAR DILATION TO FLICKER LIGHT IS REDUCED ON SHORT-TERM RE-TESTING

Jonathan E. Noonan¹ BSc(Hons) MBBS; Thanh T. Nguyen¹ MBBS PhD; Ryan E.K. Man¹ BSc; William J. Best¹ MD; Jie Jin Wang¹,² MMed PhD; Ecosse L. Lamoureux¹,³,⁴,⁵ MSc PhD.

1. Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, University of Melbourne, Melbourne, VIC, Australia
2. Centre for Vision Research, Department of Ophthalmology and Westmead Millennium Institute, University of Sydney, NSW, Australia
3. Singapore Eye Research Institute, National University of Singapore, Singapore
4. Department of Ophthalmology, National University of Singapore, Singapore
5. Duke-NUS Graduate Medical School, Singapore

Corresponding and senior author:
A/Prof Ecosse L. Lamoureux
Centre for Eye Research Australia
Department of Ophthalmology, University of Melbourne
32 Gibson Street, East Melbourne, Victoria 3002, Australia
Ph: +61 3 9929 8371
Fax: +61 3 9662-3859
Email: ecosse@unimelb.edu.au

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Key words: diabetes, flicker light, functional hyperaemia, neurovascular coupling, retinal circuitry

4.2 Abstract

**Purpose:** To investigate the impact of re-testing frequency over a short period on flicker light-induced retinal vasodilation.

**Methods:** Twenty healthy participants were included. The retinal vascular response to flicker light stimulation was assessed three times (baseline; and after five and 30 minutes of rest [tests 1, 2, and 3, respectively]) in each participant using the Dynamic Vessel Analyzer. Relative dilations of selected arteriole and venule segments during flicker stimulation and resting diameters were measured automatically. Mean vessel dilations and resting diameters were compared using repeated measures analysis of variance.

**Results:** Participants were (mean [SD]) aged 33.1 (5.7) years and mostly female (70%). Maximum arteriolar dilations during flicker stimulation were 3.2 (2.1) %, 2.4 (1.6) % and 3.4 (2.1) % in tests 1, 2, and 3, respectively. Venular dilations were 4.3 (1.3) %, 3.8 (1.6) % and 4.4 (1.7) % in the three consecutive tests. Mean arteriolar dilations were significantly different across the three tests ($P < 0.001$). Compared with the first test, arteriolar dilations were significantly reduced after five minutes ($P = 0.008$) but not 30 minutes of rest ($P = 0.44$). No significant differences were found over time for mean venular dilations ($P = 0.13$). Resting diameters of selected vessels were not significantly different between tests.

**Conclusions:** Retinal arteriolar dilation during flicker stimulation is reduced on short-term re-testing without a significant change in baseline vessel diameter, indicating decreased responsiveness to the flicker stimulus. Researchers should allow at least 30 minutes between consecutive tests to minimise suppression of the flicker response.
4.3 Introduction

Retinal arterioles and venules dilate when stimulated with flickering light, a phenomenon described as functional hyperaemia or neurovascular coupling\(^1, 2\). This response reflects an increase in blood flow to meet the increased metabolic demands of activated retinal neurons. Vasodilation occurs within seconds of flicker light stimulation and quickly reverses upon its cessation. Transient constriction is observed in arterioles, but not venules, for several seconds after withdrawal of the stimulus.

Interest in the retinal vascular response to flicker stimulation has been generated from several cross-sectional studies that showed reduced vasodilations in people with type 1 and type 2 diabetes compared with non-diabetic controls\(^12-14\). Further, reduced vasodilations have been observed in patients with diabetes prior to the evolution of typical diabetic retinopathy lesions\(^18, 19\). The retinal response to flicker stimulation may therefore represent a useful marker of retinal function in these patients.

Initial work demonstrated good reproducibility of the flicker response in healthy people across ethnic groups\(^30, 31\). However, these studies incorporated a rest period of at least 30 minutes between consecutive tests to allow sufficient recovery of retinal function. Anecdotal evidence from our Department suggests that test participants experience a transient purple discoloration of central vision following measurement of the retinal flicker response. It is unclear if this visual disturbance may represent a short-term disturbance in flicker light-activated retinal pathways.

The purpose of this study was to investigate whether retinal vasodilation during flicker stimulation is reduced on re-testing after five and 30 minutes of rest compared with baseline tests. We hypothesised that flicker-induced responses would be reduced for several minutes after the test but would recover by 30 minutes.

4.4 Methods

4.4.1 Participants

We conducted a repeated measures observational study of retinal vascular responses to flicker light stimulation on the right eye of 20 healthy volunteers aged at least 18 years recruited from the Centre for Eye Research Australia (Melbourne, Australia). Exclusion criteria were smoking, any self-reported medical conditions, eye pathology other than a need for glasses or contact lenses and tropicamide sensitivity. All participants received measurement of height, weight, blood pressure, heart rate and IOP to screen for any significant undiagnosed pathology. In addition, participants had their
refractive error (spherical equivalent) and BCVA measured with a LogMAR chart. Abstention from alcohol and caffeine-containing products was requested from all participants in the 12-hour period prior to the study.

The study protocol followed the tenants of the Declarations of Helsinki and received institutional review board approval (12/1094H). Informed consent was obtained from all participants after explanation of the nature and possible consequences of the study.

4.4.2 IOP and haemodynamic measurements

IOP and haemodynamic measurements were recorded at the beginning of the study. IOP was measured in the right eye after topical instillation of oxybuprocaine hydrochloride 0.4% and fluorescein sodium with a slit lamp-mounted Goldmann applanation tonometer (Haag-Streit, Bern, Switzerland). Blood pressure was recorded with a manual sphygmomanometer (Tycos, Welch Allyn, USA) and stethoscope (Littmann Master Cardiology, 3M, USA). Heart rate was calculated from palpation of the radial artery for 15 seconds.

4.4.3 Flicker light-induced retinal vasodilation

Flicker light-induced retinal vasodilation was measured in the right eye with the DVA (IMEDOS, Jena, Germany), modified to improve reliability in Asian people. The DVA is a commercially available system that permits reliable continuous recordings of retinal vessels with a minimal width of 90 μm in conscious human subjects. The system uses green light illumination (530-600 nm) to provide the greatest contrast between retinal blood vessels and the surrounding tissue. Erythrocytes within the vessels have a maximum absorbance of between 400-620 nm, whereas most surrounding tissue reflects light in this range. The reflected light is detected by a CCD camera and used to estimate the width of the vessels. Diffuse luminance flicker is delivered by the DVA system over the entire 30° visual field by way of an optoelectronic shutter that interrupts the light source with a bright-to-dark ratio of 25:1 at a frequency of 12.5 Hz. This frequency lies near to values previously shown to maximise retinal vasodilation and blood flow during flicker stimulation. The temporal resolution of the DVA system was 40 milliseconds, corresponding with 25 vessel diameter recordings per second.

Topical tropicamide was used for mydriasis and examinations were conducted in a dimly lit room. Participants focused on the tip of a fixation bar for the duration of each test while the fundus was examined under green light with an average luminance of 130
cd/m$^2$ (ILT1700 Research Radiometer, International Light Technologies, USA). A unique high contrast region of the fundus (e.g. a vessel branch) was chosen as a fixation target to allow the DVA software to track eye movements. Next, a straight superior temporal arteriolar and venular segment located between one half to two disc diameters from the optic disc margin and at least one vessel diameter from any bifurcation or neighbouring vessel was selected for analysis.

Vessel diameters were measured for 50 seconds, then during flicker stimulation for 20 seconds and a non-flicker period for 80 seconds. A flicker period of 20 seconds was chosen to allow vessels to dilate maximally without causing unnecessary distress to our participants$^{41, 42}$. The flicker/non-flicker cycle was repeated twice for a total duration of 350 seconds. The test was repeated after two minutes and 50 seconds to allow five minutes of rest between flicker periods. A third test was performed 27 minutes and 50 seconds after the second test to allow 30 minutes of rest between flicker periods. Repetition mode was used for re-tests so that the same vessel segments were used in each participant. Baseline vessel diameters were calculated automatically and expressed in measurement units (MU). Maximum vessel dilation was calculated as the percentage increase in vessel diameter relative to baseline after 20 seconds of flicker stimulation averaged over the three measurement cycles.

4.4.4 Statistical analysis

The mean resting diameters of arteriole and venule segments prior to flicker stimulation were compared across all tests. We then compared mean maximum retinal arteriolar and venular dilations relative to baseline during flicker stimulation. Group means were compared with repeated measures analysis of variance. Where means were significantly different, paired $t$-tests were used to compare re-tests with baseline tests using a Bonferroni correction factor of two. The post-hoc ICCs and 95% CI were calculated for outcomes in tests 1 and 3 using a two-way random effects model. Multiple linear regression models were used to investigate for any effect of age, sex, refractive error and the timing of tests on vessel diameters and flicker responses. Data were analysed in Stata (version 12.1; StataCorp, USA). $P$ values $< 0.05$ were considered significant.

4.5 Results

The baseline characteristics of participants are shown in Table 3. Participants were (mean [SD]) aged 33.1 (5.7) years, normotensive (systolic 108 [10] mmHg;
diastolic 67 [12] mmHg), with normal IOP (15 [3] mmHg) and were mostly female (70%) and Caucasian (75%). Mild myopia was present (-1.35 [2.05] spherical equivalent) but BCVA was excellent (-0.03 [0.06] with the LogMAR chart).

The diameters of selected arteriole and venule segments prior to flicker stimulation are shown in Error! Reference source not found.. Mean (SD) resting arteriole segment diameters were 114 (19) MU, 114 (18) MU and 114 (19) MU in tests 1, 2 and 3, respectively. Resting venule segment diameters were 135 (13) MU, 136 (12) MU and 135 (12) MU in the three consecutive tests. No significant differences were observed between the mean resting arteriolar (P = 0.99) or venular (P = 0.25) diameters in the three tests.

The maximum percentage dilations of selected arteriole and venule segments during flicker stimulation are illustrated in Figure 9. Mean (SD) arteriolar dilations were 3.2 (2.1) %, 2.4 (1.6) % and 3.4 (2.1) % in tests 1, 2 and 3, respectively (P < 0.001). Compared with the first test, arteriolar dilations were significantly reduced after five minutes (P = 0.008) but not 30 minutes of rest (P = 0.44) with α = 0.025 adjusted for multiple comparisons. The corresponding values for venular dilations were 4.3 (1.3) %, 3.8 (1.6) % and 4.4 (1.7) %, respectively. Mean venular dilations were however not significantly different across the three tests (P = 0.13).

The reproducibility of measures between tests 1 and 3 was assessed. The ICCs (95% CI) for resting arteriolar and venular diameters were 0.99 (0.98, 1.00) and 0.97 (0.93, 0.99), respectively. The ICCs for arteriolar and venular dilations were 0.94 (0.87, 0.98) and 0.56 (0.16, 0.80), respectively.

Age, sex, refractive error and timing of the tests were investigated for a potential effect on retinal vessel diameters and flicker responses. None of these parameters were significantly associated with baseline vessel diameters or flicker responses or changes in these outcomes between baseline and follow-up tests.

4.6 Discussion

This is the first study to show that the responsiveness of retinal arterioles to flicker stimulation is reduced in the immediate post-test period. This effect is short lived and appears to last less than 30 minutes, which would account for the good reproducibility of the retinal flicker response reported in other studies. The reproducibility of our baseline test results was excellent after 30 minutes of rest with the exception of venular dilation, which had a high degree of variability. Although mean
venular dilations were reduced after five minutes of rest, they were not statistically different.

No differences were observed in the baseline diameters of selected arteriolar or venular segments over our three tests. This indicates that retinal vessels rapidly return to their resting state on cessation of the flicker stimulus, and our results were not confounded by residual dilation from preceding tests. Further, it implies that impaired arteriolar responsiveness in the immediate post-test period may be due to desensitisation of the pathways that mediate the retinal flicker response.

One possible explanation for reduced arteriolar dilation in the immediate post-test period is a desensitisation of cones, particularly the M-cones most sensitive to the green light used in our experiments. Rods were unlikely to be significant mediators of the retinal flicker response in our experiments, as rod signalling is suppressed during constant bright light illumination\textsuperscript{255}. Although cones are resistant to visual pigment bleaching at high light intensities\textsuperscript{255}, cone sensitivity is dependent on background light intensity\textsuperscript{256}. Light adaptation from prolonged fundus illumination might therefore have reduced cone signalling in our second test of the flicker response. Further studies are required to determine whether short-term impaired arteriolar responses involve light adaptation in cone photoreceptors.

In the primate visual system, flickering light activates magnocellular and parvocellular neural pathways to varying degrees according to parameters such as the stimulus frequency and the degree of luminance or chromatic modulation\textsuperscript{64, 257}. Magnocellular retinal ganglion cells are activated by central and surrounding light impulses and respond optimally to luminance modulation at frequencies of 10-20 Hz\textsuperscript{258}. In contrast, parvocellular ganglion cells receive opposing input from different wavelength-specific cones and are most sensitive to chromatic modulation at frequencies of 5 Hz or less\textsuperscript{258-260}. Luminance flicker with red or green light produces the greatest increase in optic nerve head blood flow at frequencies of between 10 to 15 Hz\textsuperscript{90}. The magnocellular pathway is probably the major driver of this response, with a lesser contribution from the parvocellular pathway\textsuperscript{258, 261}. Chromatic flicker with minimal or no luminance change produces a maximal increase in optic nerve head blood flow and retinal vasodilation with red-green modulation at a frequency of approximately 2 Hz\textsuperscript{42, 90}. Low frequency equiluminant chromatic flicker is thought to be selective for the parvocellular pathway\textsuperscript{90, 258}.  

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Flicker stimulation in our study was delivered by green light at a frequency of 12.5 Hz at a modulation depth of approximately 1°, corresponding with pure luminance modulation. These stimulus parameters are most selective for the magnocellular pathway with a possible lesser influence on the parvocellular pathway. The reduced arteriolar response to luminance flicker stimulation in our second test may reflect decreased neural activity, particularly through magnocellular retinal ganglion cells. Tests of ganglion cell function or cortical visual processing such as the PERG or VEP, respectively, may help to identify whether reduced short-term arteriolar responses reflect an impairment at the level of retinal neurons or vascular cells. It is also worth considering whether reduced flicker light-induced retinal vasodilation in patients with diabetes may reflect dysfunctional retinal processing through the magnocellular or parvocellular neural pathways12-14.

The mechanisms by which flicker light causes retinal vasodilation remain unclear, although nitric oxide has been implicated as an important signalling molecule. Systemic inhibition of NOS with L-NMMA reduced the retinal flicker response by about half in healthy volunteers7. Further, flicker stimulation increases blood flow to the optic nerve head and retina via nitric oxide in the cat5, 6. Some researchers have interpreted these findings to mean that the retinal flicker response is a measure of endothelial function4, 13. However, no study has convincingly identified the NOS isoform(s) activated during flicker stimulation or the cellular source of nitric oxide. It is possible that retinal neurons activate neuronal NOS during flicker stimulation and directly mediate vasodilation without a major contribution from endothelial cells. In addition, rodent studies suggest that retinal glial cells are important mediators of the flicker response via the release of vasodilatory arachidonic acid-derived epoxyeicosatrienoic acids and prostaglandins32, 33. Clearly, more work is required to elucidate the relative contributions of retinal neurons, glia and vascular cells to the retinal flicker response.

Strengths of our study include our relatively young and healthy participant sample, which included both Asian and Caucasian participants. Measurement of the retinal flicker response is generally more difficult in Asians with current technology due to their narrow set eyes31, but this did not preclude our significant findings. In addition, the simple repeated measures design of our study provided a precise means to investigate the impact of short-term re-testing on the retinal flicker response.
Limitations of our study include a relatively small sample size, which may have been underpowered to detect a significant reduction in venular dilation on re-test. Second, we did not specifically exclude people with myopia because of our focus on within-subject changes, although high myopia could potentially affect the flicker response. This is unlikely to be a major confounder of our results though due to the mild myopia and excellent BCVA in our sample. Third, our equipment could only test the flicker response with green light specific for M-cones. The addition of more light wavelengths may yield larger vasodilations but could make accurate measurements more difficult. Fourth, given that all our participants were healthy it is unclear whether diabetes or diabetic retinopathy might affect the length of time needed to recover from flicker stimulation. Last, our study is unable to provide specific information on the mechanism of impaired arteriolar dilation in the immediate post-test period.

Follow-up studies should be undertaken to investigate why retinal arteriolar dilation during diffuse luminance flicker stimulation was reduced on re-test with the same parameters after five minutes of rest. For example, the influence of light adaptation could be investigated by testing the flicker response after a period of exposure to specific background luminance levels. Conventional ERG parameters could be investigated before and after tests of the flicker response. Higher processing of visual signals could be investigated; if retinal arteriolar dilation was reduced due to desensitisation of local neural pathways we would expect to see a similar reduction in the VEP amplitude. Given that our flicker parameters were relatively specific for the magnocellular pathway, the effect of low frequency equiluminant chromatic flicker directed at the parvocellular pathway should also be investigated in future studies. As described above, vessel diameters are best measured under green light so chromatic flicker should use alternating green-red or green-blue light while the luminance level is held constant.

Our finding of reduced arteriolar responsiveness in the immediate post-test period has important implications for the measurement of the retinal flicker response in clinical studies. If this response is to be used as a marker of retinal function in patients with diabetes then other sources of variation need to be minimised. Researchers using the DVA machine should allow at least 30 minutes between consecutive tests where indicated, and minimise the duration of fundus illumination.
4.7 Acknowledgements

The Centre for Eye Research Australia receives Operational Infrastructure Support from the Victorian Government. JN is supported by the Australian NHMRC (National Health and Medical Research Council) Postgraduate Medical Scholarship (ID1038701). Ecosse Lamoureux is supported by the Australian NHMRC Senior Research Fellowship (ID1045280).
Table 3. Baseline characteristics of participants.

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Data are expressed as means (SD) unless otherwise indicated.

Figure 8. Baseline arteriolar and venular diameters prior to flicker stimulation in the first and subsequent tests with five and 30 minutes of rest. Data are means ± standard error of the mean (SEM).
Figure 9. Maximum arteriolar and venular dilation during flicker stimulation in the first and subsequent tests with five and 30 minutes of rest. Data are means ± SEM. ** $P < 0.01$ vs. the first test.
CHAPTER 5. FLICKER-INDUCED RETINAL ARTERIOLE DILATION IS REDUCED BY AMBIENT LIGHTING
5.1 Context to the paper

This paper is presented in lieu of a traditional thesis chapter. It addresses Aim 2 of my doctoral project, “To investigate the effect of ambient lighting on luminance flicker-induced retinal vasodilations in healthy people”. It has been lightly edited from its published version for Australian spelling, presentation and consistency within this doctoral thesis. The citation is:


The nature and proportion of my contributions and those of my collaborators to the work presented in this chapter are described in the Preface of this thesis.
FLICKER-INDUCED RETINAL ARTERIOLE DILATION IS REDUCED BY AMBIENT LIGHTING

Jonathan E. Noonan¹ BSc(Hons) MBBS; Gregory J. Dusting¹ PhD FBPharmacolS; Thanh T. Nguyen¹ MBBS PhD; Ryan E. K. Man¹,² BSc PhD; William J. Best¹ MD; Ecosse L. Lamoureux¹-³ MSc PhD.

1. Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, University of Melbourne, Melbourne, VIC, Australia
2. Singapore Eye Research Institute and Department of Ophthalmology, National University of Singapore, Singapore
3. Duke-NUS Graduate Medical School, Singapore

Corresponding and senior author:
A/Prof Ecosse L. Lamoureux
Centre for Eye Research Australia
Department of Ophthalmology, University of Melbourne
32 Gisborne Street, East Melbourne, Victoria 3002, Australia
Ph: +61 3 9929 8371
Fax: +61 3 9662-3859
Email: ecosse@unimelb.edu.au

Disclosures: Nothing to disclose.

Key words: flicker light, functional imaging, neurovascular coupling, retinal blood flow

Word count: Abstract 243; Manuscript (ex Abstract) 2,785.
5.2 Abstract

**Purpose:** To investigate the impact of ambient room lighting on the magnitude of flicker light-induced retinal vasodilations in healthy individuals.

**Methods:** Twenty healthy non-smokers participated in a balanced 2 x 2 crossover study. Retinal vascular imaging was performed with the Dynamic Vessel Analyzer under reduced or normal ambient lighting, then again after 20 minutes under the alternate condition. Baseline diameters of selected arteriole and venule segments were recorded in measurement units (MU). Maximum percentage dilations from baseline during 20 seconds of luminance flicker were calculated from the mean of three measurement cycles. Within-subject differences were assessed by repeated measures analysis of variance with the assumption of no carryover effects and pairwise comparisons from the fitted model.

**Results:** Mean (SD) maximum arteriole dilations during flicker stimulation under reduced and normal ambient lighting were 4.8 (2.3) % and 4.1 (1.9) %, respectively ($P = 0.02$). Maximum arteriole dilations were (mean ± 95% CI) 0.7 ± 0.6 % lower under normal ambient lighting compared with reduced lighting. Ambient lighting had no significant effect on maximum venular dilations during flicker stimulation or on the baseline diameters of arterioles or venules.

**Conclusions:** Retinal arteriole dilation in response to luminance flicker stimulation is reduced under higher ambient lighting conditions. Reduced responses with higher ambient lighting may reflect reduced contrast between the ON and OFF flicker phases. Although it may not always be feasible to conduct studies under reduced lighting conditions, ambient lighting levels should be consistent to ensure that comparisons are valid.
5.3 Introduction

Retinal blood vessels dilate when the retina is stimulated with flickering light\textsuperscript{262}. This phenomenon is thought to reflect an increase in blood flow to meet the increased metabolic needs of activated neurons. The precise mechanism is not completely understood but is thought to involve increased ganglion cell activity\textsuperscript{1} and local nitric oxide production\textsuperscript{5-7}.

Repetitive modulation of the luminance or chromatic characteristics of a light source produces an increase in retinal blood flow. Luminance modulation is relatively selective for the magnocellular visual pathway, derived from parasol ganglion cells, whereas chromatic modulation is selective for the parvocellular pathway and midget ganglion cells\textsuperscript{258}. Parasol ganglion cells receive mixed input from L- (red) and M-cones (green) in their receptive fields and carry achromatic visual information. In contrast, midget cells receive L- and M-cone antagonistic input and mediate red-green colour opponency\textsuperscript{60, 61, 263}. Maximal luminance or chromatic flicker-induced retinal hyperaemia occurs when the flicker phases are directed at opposing ganglion cell populations\textsuperscript{90}.

Flicker light-induced retinal vasodilation has received particular attention as a marker of retinal function in diabetes since the development \textit{in vivo} imaging techniques. For example, studies have consistently found that people with diabetes have significantly reduced responses compared with their non-diabetic counterparts\textsuperscript{12-14}, even in the absence of diabetic retinopathy\textsuperscript{18, 19}. Measurement of retinal vasodilation during flicker stimulation may therefore represent a sensitive biomarker of retinal damage in diabetes.

Measurement of retinal vasodilation during flicker stimulation is generally conducted under reduced ambient lighting conditions\textsuperscript{13, 14, 262}. However, it may not always be feasible to examine people in the dark, such as when examinations must be conducted in a room with limited ability to reduce the ambient lighting, or when other procedures or monitoring dictate that good lighting is required. It is unclear whether ambient lighting could affect the magnitude of retinal vasodilations during flicker stimulation. If true, direct comparisons between tests conducted under different ambient lighting conditions would not be valid.

The purpose of this study was to investigate whether flicker light-induced retinal arteriole or venule dilation is affected by ambient lighting. We hypothesised that tests conducted under reduced ambient lighting would yield the same results as those conducted under normal room lighting. In addition, we hypothesised that baseline
retinal arteriole and venule diameters would be unchanged between reduced and normal ambient lighting conditions.

5.4 Methods
5.4.1 Participants

We studied the right eyes of 20 healthy non-smokers aged at least 18 years recruited from the Centre for Eye Research Australia (Melbourne, Australia). Exclusion criteria were any self-reported systemic condition, epilepsy, significant eye pathology other than a need for glasses or contact lenses, and tropicamide sensitivity. Participants were not excluded on the basis of myopia. However, one participant with a refractive error (spherical equivalent) of −6.75 was excluded because we could not identify an arteriole segment near the optic disc greater than 90 MU wide. This is the limit of accuracy for dilation measurements with the DVA (IMEDOS, Jena, Germany). All participants underwent measurement of their BCVA, refractive error (spherical equivalent), IOP, blood pressure, heart rate and body mass index (BMI).

The study protocol followed the tenants of the Declaration of Helsinki and received institutional review board approval (12/1094H). Informed consent was obtained from all participants after explanation of the nature and possible consequences of the study.

5.4.2 IOP and haemodynamic measurements

The IOP was measured in the right eye after topical instillation of oxybuprocaine hydrochloride, 0.4%, and fluorescein sodium with a slitlamp-mounted Goldmann applanation tonometer (Haag-Streit, Bern, Switzerland). The blood pressure and heart rate were recorded after five minutes in a seated position with an automatic upper arm sphygmomanometer (HEM-7000-C1L, Omron Healthcare, Lake Forest, IL).

5.4.3 Crossover study

Flicker light-induced retinal vasodilation was measured in two periods with at least 20 minutes between consecutive periods. We previously found that sensitivity of the retinal arteriole response to flicker light stimulation recovers within five to 30 minutes, probably as a result of adaptation in cones after withdrawal of the light source. We chose a rest period of 20 minutes for the present study, given that dark adaptation in cones after exposure to bright light is complete within five to 10 minutes. Tests were conducted under reduced ambient lighting and normal ambient lighting. Normal ambient lighting was with three sets of two ceiling-mounted 1.2 meter-
long 36 watt fluorescent lights positioned approximately two meters above the equipment. Reduced ambient lighting was with these lights turned off. Ten participants were tested under reduced followed by normal ambient lighting (sequence 1), while 10 participants were tested under normal followed by reduced ambient lighting (sequence 2).

5.4.4 Flicker light-induced retinal vasodilation

We measured flicker light-induced retinal vasodilation in the right eye after dilation with topical tropicamide 1% with the DVA (IMEDOS, Jena, Germany) as described previously. Briefly, participants were seated and instructed to fixate on the tip of a fixation bar inside the camera while the fundus was examined under green light (530-600 nm) with the mydriatic camera (FF450plus, Carl Zeiss AG, Jena, Germany) set to a viewing angle of 30°. The light source was set to an average luminance of 130 cd/m² for both ambient lighting conditions, measured with an ILT1700 Research Radiometer (International Light Technologies, Peabody, USA). A unique high-contrast region (e.g. a vessel branch) was selected as a fixation target to allow the DVA software (IMEDOS, Jena, Germany) to compensate for eye movements and blinking. Next, we selected for analysis a straight segment of a temporal arteriole and venule located between one half to two disc diameters from the optic disc margin and at least one vessel diameter from any bifurcation or neighbouring vessel. Superior vessels were chosen where possible to reduce interference from the upper eyelid. An example of vessel selection is provided in Figure 10.

Vessel diameters were automatically and continuously measured in real-time for 350 seconds. This consisted of 50 seconds of constant light, followed by three cycles of 20 seconds of diffuse luminance flicker at 12.5 Hz and 80 seconds of constant light. Repetition mode was used for the second test to ensure that the same vessel segments were studied. If repetition mode was unable to automatically re-identify the vessel segments, the same segments were manually re-selected using an image of the previous examination location. The distance of the camera from the participant was adjusted during the test to maintain the software brightness indicator between 30-40% on an arbitrary scale from 0-100%. Baseline vessel diameters were reported in MU, where 1 MU is equivalent to 1 µm for the Gullstrand eye. Maximum vessel dilation was calculated from the mean of the three measurement cycles as the maximum percentage increase in vessel diameter relative to baseline during 20 seconds of flicker stimulation.
In addition, the time of maximum dilation (sec.) and the AUC during flicker stimulation (% x sec.) was measured for each participant.

Retinal illumination was estimated with an EasyView Digital Light Meter (EA30, Extech Instruments, USA) placed at the location of the eye. Illumination was 170 lux and 0.1 lux during the ON and OFF flicker phases under reduced ambient lighting, respectively. Illumination under normal ambient lighting was 190 lux and 30 lux during the ON and OFF flicker phases, respectively. This corresponded to a modulation depth of 1.00 and 0.73 under reduced and normal ambient lighting, respectively. The modulation depth was calculated by the Michelson formula as \((I_1 - I_2)/(I_1 + I_2)\), where \(I_1\) and \(I_2\) represent the illuminance during the ON and OFF flicker phases, respectively.\(^{90}\)

5.4.5 Statistical analyses

The maximum dilations during flicker stimulation and the baseline diameters of retinal arteriole and venule segments were compared by ANOVA. Sequence (the order of lighting conditions) was the between-subject factor. Period and lighting were the within-subject factors. This method determined whether our results were impacted by: 1) the order of lighting conditions; 2) the chronological order of the tests; and 3) ambient lighting. A technical limitation of the 2 x 2 crossover study is that carryover effects cannot be assessed with the other three factors, but our study was designed to minimise such effects. Pairwise comparisons of the dependent variables between the alternative lighting conditions were performed from the fitted ANOVA model. Data were analysed in STATA (version 12.1; StataCorp LP, College Station, TX). \(P < 0.05\) was considered significant. Based on previous data\(^{262}\), our crossover study with 20 participants would have a power of 85% to detect a 0.5% difference in mean arteriole dilations at the 0.05 significance level, assuming a within-subject SD of 0.5%.

5.5 Results

The baseline characteristics of participants are described in Table 4. Participants were relatively young (age 32.8 [6.5] years), had healthy systolic (113 [13] mmHg) and diastolic (75 [9] mmHg) blood pressures, and had excellent vision (BCVA -0.1 [0.1] by LogMAR chart). Participants were generally emmetropic or mildly myopic (spherical equivalent -0.9 [1.8] dioptries) and most were female (75%).

The mean arteriole and venule responses over time under reduced and normal ambient lighting are expressed graphically in Figure 11. Maximum dilations and their
timings, the AUC of vessel responses and baseline diameters under reduced and normal ambient lighting conditions are summarised in Table 5. Mean (SD) maximum arteriole dilations under reduced and normal ambient lighting were 4.8 (2.3) % and 4.1 (1.9) %, respectively ($P = 0.02$). The corresponding values for venule dilations were 5.4 (1.8) % and 5.1 (2.1) %, respectively ($P = 0.40$). Maximum arteriole dilations were (mean ± 95% CI) 0.7 ± 0.6 % lower under normal ambient lighting compared with reduced lighting. In addition, the AUC of arteriole responses were reduced by 8.4 ± 7.1 % x sec. Ambient lighting had no significant effect on the AUC of the venule response or the timing of maximum vessel dilations during flicker stimulation. The maximum relative dilations of retinal arterioles and venules during flicker stimulation under the two different ambient lighting conditions are expressed graphically in Figure 12. No significant sequence or period effects were identified for maximum arteriole or venule dilations.

The baseline diameters of selected retinal arteriole and venule segments are shown in Figure 13. Mean (SD) baseline arteriole diameters under reduced and normal ambient lighting were 123 (16) MU and 123 (16) MU, respectively. Baseline venule diameters under reduced and normal ambient lighting were 150 (15) MU and 151 (14) MU, respectively. Ambient lighting had no significant effect on baseline arteriole or venule diameters. Further, no significant sequence or period effects were identified for baseline arteriole or venule diameters.

5.6 Discussion

The results of our study indicate that retinal arteriole dilations during luminance flicker stimulation are smaller when tests are conducted under higher ambient lighting conditions, in contrast to our original hypothesis. Any effect of ambient lighting on venule dilations appears to be negligible. As predicted, we found no suggestion that ambient lighting affects baseline retinal arteriole or venule diameters. This result is important, as it shows that comparisons of flicker light-induced retinal vasodilations are only valid when tests are conducted under the same ambient lighting conditions.

Retinal vasodilation during flicker stimulation is an indirect marker of neuronal activity-dependent hyperaemia. However, it is currently more reliable than direct measures of hyperaemia, such as with laser Doppler velocimetry, which are unable to take real-time measurements during flicker stimulation and produce more variable results$^{19, 262}$. According to the Hagen-Poiseuille equation, laminar flow through a
cylinder is proportional to the fourth power of the radius. A small increase in the radius of a blood vessel therefore leads to a relatively large increase in blood flow. For these reasons, vasodilation is generally considered to be an acceptable surrogate marker of hyperaemia.

It is curious that reduced arteriole dilations during flicker stimulation under normal ambient lighting were not associated with a similar reduction in venule dilations, given that blood flow through the arterial and venous circulations should match. We previously observed reduced arteriole dilations during flicker stimulation on short-term retesting, probably due to light adaptation over the course of the test. As with the present study, we did not observe a significant effect on venule dilations. In addition, we found that while arteriole dilations had excellent reproducibility, venule dilations were less consistent. Blood flow is dependent on vessel diameter and blood velocity. It is likely that arteriole flow is strongly related to arteriole diameter, whereas blood velocity, which is not measured by the DVA, may be a more important determinant of venule flow.

Retinal ganglion cells are thought to be the main neuronal mediators of hyperaemia during flicker stimulation. The two main ganglion cells in the primate retina are parasol cells, which give rise to the magnocellular pathway, and midget cells of the parvocellular pathway. These cells are activated by flickering light to different degrees according to characteristics such as the flicker frequency, modulation depth, luminance contrast and chromatic contrast. Parasol cells are most sensitive to luminance contrast modulation at frequencies between 10-20 Hz, whereas midget cells are most sensitive to chromatic contrast modulation at frequencies below 5 Hz. Our flicker stimulus had a constant wavelength of 530-600 nm at a frequency of 12.5 Hz. Thus, our stimulus was most selective for parasol cells of the magnocellular pathway.

With some exceptions, ganglion cells have a central receptive field with a concentrically arranged antagonistic surround. For luminance signals, both parasol and midget cells can be either ON centre/OFF surround or OFF centre/ON surround. Parasol cells receive mixed input from L- and M-cones in both areas. Midget cells receive overlapping red-green opponent signals and are either L-ON/M-OFF or L-OFF/M-ON. ON centre midget cells receive a dominant ON signal from L- or M-cones in the centre, whereas OFF centre midget cells receive a dominant OFF signal from L- or M-cones in the centre. S-cone signals are carried by small bistratified ganglion cells and do not appear to involve the magnocellular or parvocellular pathways. Luminance flicker
with green light at a frequency of 12.5 Hz, as in our study, might therefore increase net ganglion cell activity by stimulating both ON and OFF centre parasol cells. Midget cells might also contribute to a small increase in ganglion cell activity despite being less sensitive to our stimulus.

An important determinant of retinal hyperaemia during luminance flicker stimulation is the modulation depth. That is, a greater change in luminance between the ON and OFF flicker phases produces a greater increase in retinal blood flow. Lower flicker light-induced arteriole dilations under higher ambient lighting could therefore be explained in terms of a reduction in the modulation depth. With higher ambient lighting, retinal luminance would remain higher during the OFF flicker phase and the stimulation of OFF centre ganglion cells would be reduced. This probably accounts for why arteriole dilations were lower under normal ambient lighting in our study.

Data from humans and cats indicate that nitric oxide is important for retinal vasodilations during flicker stimulation. Nitric oxide is synthesised by three isoforms of nitric oxide synthase: nNOS, eNOS and iNOS. Mouse data indicate that nNOS is found in retinal bipolar, amacrine and ganglion cells. Further, recent evidence indicates that nNOS is the dominant isoform in the retina and that light-induced nitric oxide production occurs primarily in the two plexiform layers away from most blood vessels. eNOS is about half as prevalent as nNOS and is primarily found in blood vessels. iNOS is not present in significant quantities under normal conditions.

Luminance flicker might increase nNOS activity in ganglion cells, with local diffusion of nitric oxide to neighbouring inner retinal blood vessels, vasodilation and increased blood flow. A smaller change in retinal luminance between flicker phases could therefore produce smaller arteriole dilations via a reduction in nitric oxide release from parasol ganglion cells.

The main strengths of this study were its simple crossover design and clear distinction between the two ambient lighting conditions under investigation. In addition, all measurements were taken by a single experienced investigator (J.N.). The reliability of our measurements meant that we had good statistical power to detect small differences in our main outcomes with a relatively small number of participants. We recognise that our study was limited in its ability to explain a reduction in arteriole dilation during flicker stimulation with higher ambient lighting. Although this may involve reduced ganglion cell activity, our study was unable to provide direct evidence to support this hypothesis. Another limitation was our over-representation of female
participants, even though we have not previously observed an effect of sex on DVA measurements\textsuperscript{262}.

Our study demonstrates that ambient lighting is an important determinant of arteriole dilation during luminance flicker stimulation. Studies of this response should therefore ensure that comparisons are only made between tests conducted under the same ambient lighting conditions. It may not always be feasible to conduct tests in the dark, but if higher ambient lighting is unavoidable it is important to maintain consistent lighting throughout each study.

5.7 Acknowledgments

The Centre for Eye Research Australia receives Operational Infrastructure Support from the Victorian Government. Project funding was received from the Royal Victorian Eye and Ear Hospital. JN was supported by the Australian NHMRC (National Health and Medical Research Council) Postgraduate Medical Scholarship (ID1038701). EL and GJD were supported by Australian NHMRC Research Fellowships (ID1045280 and 1003113).
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Data are expressed as means (SD) unless otherwise indicated.

Table 5. Dynamic imaging parameters under reduced and normal ambient lighting.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reduced lighting</th>
<th>Normal lighting</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Maximum dilations (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arterioles</td>
<td>4.8 (2.3)</td>
<td>4.1 (1.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Venules</td>
<td>5.4 (1.8)</td>
<td>5.1 (2.1)</td>
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<tr>
<td>Time of maximum dilations (sec.)</td>
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<td>Arterioles</td>
<td>15.5 (4.4)</td>
<td>13.5 (5.3)</td>
<td>0.18</td>
</tr>
<tr>
<td>Venules</td>
<td>16.4 (2.2)</td>
<td>16.9 (2.7)</td>
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<tr>
<td>AUC during flicker (% x sec.)</td>
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<tr>
<td>Arterioles</td>
<td>57.6 (30.5)</td>
<td>49.2 (28.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Venules</td>
<td>58.9 (23.2)</td>
<td>54.1 (21.2)</td>
<td>0.16</td>
</tr>
<tr>
<td>Baseline diameters (MU)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Arterioles</td>
<td>123 (16)</td>
<td>123 (16)</td>
<td>0.82</td>
</tr>
<tr>
<td>Venules</td>
<td>150 (15)</td>
<td>151 (14)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD).
Figure 10. An example of arteriole and venule segment selection.

Figure 11. Mean relative dilations over time under reduced and normal ambient lighting. A: arterioles. B: venules.
Figure 12. Maximum relative retinal arteriole and venule dilations during flicker stimulation. A: Responses under reduced and normal ambient lighting. B: Differences in individual responses under normal compared with reduced ambient lighting. Data are means ± SEM. *P < 0.05.

Figure 13. Baseline retinal arteriole and venule diameters. A: Diameters under reduced and normal ambient lighting. B: Differences in individual diameters under normal compared with reduced ambient lighting. Data are means ± SEM.
CHAPTER 6. FLICKER LIGHT-INDUCED RETINAL VASODILATION IS UNAFFECTED BY INHIBITION OF EPOXYEICOSATRIENOIC ACIDS AND PROSTAGLANDINS IN HUMANS
6.1 Context to the paper

This paper is presented *in lieu* of a traditional thesis chapter. It addresses **Aim 3** of my doctoral project, “To investigate the role of EETs and PGs in luminance flicker-induced retinal vasodilation in healthy people”. It has been lightly edited from its published version for Australian spelling, presentation and consistency within this doctoral thesis. The citation is:


The nature and proportion of my contributions and those of my collaborators to the work presented in this chapter are described in the Preface of this thesis.
FLICKER LIGHT-INDUCED RETINAL VASODILATION IS UNAFFECTED BY INHIBITION OF EPOXYEICOSATRIENOIC ACIDS AND PROSTAGLANDINS IN HUMANS

Jonathan E. Noonan¹ BSc(Hons) MBBS; Gregory J. Dusting¹ PhD FBPharmacolS; Thanh T. Nguyen¹ MBBS PhD; Alicia J. Jenkins²-⁴ MBBS MD FRACP; Ryan E. K. Man¹ PhD; William J. Best¹ MD; Daniel A. Dias⁵ PhD; Nirupama S. Jayasinghe⁵ BSc(Hons); Ute Roessner⁵ PhD; Ecosse L. Lamoureux¹,⁶,⁷ MSc PhD.

1. Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, University of Melbourne, Melbourne, VIC, Australia
2. Department of Medicine, St. Vincent's Hospital, University of Melbourne, Melbourne, VIC, Australia
3. NHMRC Clinical Trials Centre, University of Sydney, Sydney, NSW, Australia
4. Queens University, Belfast, N. Ireland. UK.
5. Metabolomics Australia, School of Botany, University of Melbourne, Parkville, VIC, 3010, Australia
6. Singapore Eye Research Institute and Department of Ophthalmology, National University of Singapore, Singapore
7. Duke-NUS Graduate Medical School, Singapore

Corresponding and senior author:
A/Prof Ecosse L. Lamoureux
Centre for Eye Research Australia
Department of Ophthalmology, University of Melbourne
32 Gisborne Street, East Melbourne, Victoria 3002, Australia
Ph: +61 3 9929 8371
Fax: +61 3 9662-3859
Email: ecosse@unimelb.edu.au

Disclosures: Nothing to disclose.

Key words: epoxyeicosatrienoic acids, functional imaging, neurovascular coupling, prostaglandins, retinal blood flow

Word count: Abstract 246; Manuscript (ex Abstract) 3,423.
6.2 Abstract

**Purpose:** To investigate the role of epoxyeicosatrienoic acids (EETs) and prostaglandins (PGs) in retinal blood vessel diameters and vasodilation during flicker light stimulation in humans.

**Methods:** Twelve healthy non-smokers participated in a balanced crossover study. Oral fluconazole 400 mg and dispersible aspirin 600 mg were used to inhibit production of EETs and PGs, respectively. Retinal imaging was performed one hour after drug ingestion with the Dynamic Vessel Analyzer. Resting diameters of selected vessel segments were recorded in measurement units (MU). Maximum percentage dilations during flicker stimulation were calculated from baseline diameters. We then studied six participants each after fluconazole and aspirin ingestions at 30-minute intervals for two hours. Within-subject differences were assessed by analysis of variance and Dunnett-adjusted pairwise comparisons with significance taken at \( P < 0.05 \).

**Results:** In crossover study participants, mean (SD) arteriole and venule dilations without drug administration were 4.4 (2.0) % and 4.6 (1.7) %, respectively. Neither drug affected vasodilation during flicker stimulation. Mean (SD) resting arteriole and venule diameters on no-drug visits were 120 (11) MU and 146 (17) MU, respectively. Fluconazole reduced mean ± 95% CI resting venule diameters by 5 ± 4 MU. In repeated measures participants, neither drug affected vasodilations, but fluconazole reduced resting venule diameters over two hours \( P < 0.001 \).

**Conclusions:** EETs and PGs are unlikely to be primary mediators of flicker light-induced retinal vasodilation in humans. However, EETs may play a role in the regulation of retinal vascular tone and blood flow under resting physiological conditions.
6.3 Introduction

Retinal vasodilation, a surrogate marker of hyperaemia, is a normal physiological response to flicker light stimulation. The increased blood flow provides additional oxygen and nutrients to meet the increased requirements of metabolically active cells. Retinal hyperaemia in response to flicker light is thought to be a function of ganglion cell activity\textsuperscript{1} and nitric oxide release\textsuperscript{5-7}. However, the exact mechanism of increased retinal blood flow during flicker light stimulation in health remains unclear, which makes the significance of changes in the response during disease difficult to understand.

Experimental data from rodents challenges the view that nitric oxide is a key mediator of retinal neuronal activity-dependent hyperaemia. Using \textit{ex vivo} rat retinas perfused with 95% oxygen, Metea and Newman\textsuperscript{32} found that EETs were important mediators of flicker light-induced arteriolar dilations. Although PGs did not contribute to flicker light-induced arteriolar dilations during hyperoxia, PGs did appear to mediate dilations in retinas perfused with 21% oxygen\textsuperscript{33}. Interestingly, increasing the background nitric oxide concentration caused arterioles to constrict during flicker light stimulation\textsuperscript{32}. Flicker light-induced arteriolar dilations were inhibited by blockade of neuron-to-glia signalling and were replicated by direct glial cell stimulation, suggesting that these vasomotor responses were mediated by glial-derived EETs and PGs\textsuperscript{32, 33}. Of the dilatory PGs, glial cells and neurons generally produce PGE\textsubscript{2}\textsuperscript{3}.

The \textit{in vivo} production of EETs and PGs can be inhibited by fluconazole and aspirin, respectively. Fluconazole, an anti-fungal drug, is a potent inhibitor of CYP2C enzymes\textsuperscript{112}. Fluconazole was previously used to demonstrate EET involvement in the control of radial artery tone and flow-mediated dilation in humans\textsuperscript{113, 114}. Aspirin, an irreversible inhibitor of COX enzymes\textsuperscript{115, 116}, has similarly been used to show that PGs can mediate cutaneous vasodilations\textsuperscript{117, 118} or vasoconstrictions\textsuperscript{119} in response to pharmacological stimuli.

We have now investigated whether EETs and PGs are important mediators of flicker light-induced retinal vasodilation or affect retinal vascular tone in humans. We hypothesised that fluconazole and aspirin would reduce the magnitude of flicker light-induced vasodilations, but have a negligible impact on resting retinal vascular diameters.
6.4 Methods

6.4.1 Participants

In a balanced crossover study we studied the right eyes of 12 healthy adults (aged ≥18 years) who reported no chronic medical conditions. Exclusion criteria were smoking, eye surgery other than refractive surgery, allergies to study drugs, regular medications other than dietary supplements, pregnancy and lactation. Additional exclusion criteria were: 1) any ocular pathology detected on two-field fundus photography of the right eye centred on the macula and optic disc with a 45° digital non-mydriatic camera (CR-2, Canon, Melville, NY); 2) any abnormality detected on a 12-lead electrocardiogram (ELI 150 Rx, Mortara Instrument, Milwaukee, WI); and 3) any major abnormalities found during a general clinical examination by a medical professional (JN). All participants had their age, sex, ethnicity, height and weight, heart rate, blood pressure, iris pigmentation and IOP, recorded at baseline. Abstention from alcohol and caffeine on study days was requested.

After conclusion of the crossover study, we took repeated measurements of flicker light-induced retinal vasodilation after fluconazole and aspirin ingestions using the right eyes of six participants per drug. Recruitment criteria and baseline measurements for these repeated measures studies were identical to the crossover study. Participants were eligible for any or all studies provided that they met all of the inclusion and exclusion criteria at baseline and had a washout period of at least two weeks between studies.

This study followed the tenets of the Declaration of Helsinki and was approved by the Royal Victorian Eye and Ear Hospital institutional review board (12/1094H). Written informed consent was obtained from all participants.

6.4.2 IOP and haemodynamic measurements

The right eye IOP was measured after topical oxybuprocaine hydrochloride, 0.4% using fluorescein sodium with a slitlamp-mounted Goldmann applanation tonometer (Haag-Streit, Bern, Switzerland). The baseline blood pressure and heart rate were recorded in a seated position after approximately five minutes’ rest with an automatic upper arm sphygmomanometer (HEM-7000-C1L, Omron Healthcare, Lake Forest, IL).

6.4.3 Crossover study

Participants were seen on three separate days at least two weeks apart. At each visit, participants received no drug (control), oral fluconazole 400 mg (Sandoz,
Pyrmont, Australia) or dispersible aspirin 600 mg (Reckitt Benckiser, West Ryde, Australia). The drug order was randomised according to Table 6. A single 400 mg oral dose of fluconazole was expected to produce a plasma concentration of \( \approx 6.72 \mu g/ml \) at one hour post-ingestion\(^{267} \), similar to the plasma concentration used to implicate EETs in radial artery haemodynamics\(^{113,114} \). A single 600 mg dose of dispersible aspirin was expected to produce a plasma salicylate concentration of \( \approx 40 \mu g/ml \) at one hour post-ingestion\(^{268} \).

At each visit, participants had their right eye dilated (topical tropicamide, 1%), and had a 20G cannula inserted in an antecubital fossa vein for blood sampling. Participants were seated in a quiet area for 30 minutes after cannulation. Flicker light-induced retinal vasodilation was measured at each visit and one hour after drug ingestions to allow time for absorption. Venous blood was collected in EDTA tubes and centrifuged at the time of the functional imaging. Plasma was stored (-80°C) until analyses.

6.4.4 Repeated measures studies

Similar to the main crossover study, participants received oral fluconazole 400 mg or dispersible aspirin 600 mg and the right eye was dilated with topical tropicamide, 1%. However, participants in these repeated measures studies were not cannulated. Flicker light-induced retinal vasodilation was measured at baseline and at 30-minute intervals for two hours after drug ingestion. We have previously shown that 30 minutes between tests is sufficient to allow retinal flicker response recovery\(^{262} \).

6.4.5 Flicker light-induced retinal vasodilation

Flicker light-induced retinal vasodilation was measured with the DVA (IMEDOS, Jena, Germany) as described previously\(^{262} \). Briefly, participants seated in a dimly lit room were instructed to fixate on the tip of a fixation bar inside the camera while the fundus was examined under green light (\( \lambda = 530-600 \) nm) with the mydriatic camera (FF450plus, Carl Zeiss AG, Jena, Germany) set to a viewing angle of 30°. The light source was set to an average luminance of 130 cd/m\(^2\) for all tests, measured with an ILT1700 Research Radiometer (International Light Technologies, Peabody, USA). A unique fundus location was chosen as a tracking target for the DVA software (IMEDOS, Jena, Germany). Next, we selected a straight temporal arteriole and venule segment one half to two disc diameters from the optic disc margin and at least one
vessel diameter from any bifurcation or neighbouring vessel. Superior vessels were chosen where possible to reduce upper eyelid interference.

Vessel diameters were automatically and continuously measured in real-time for 350 seconds. This consisted of 50 seconds of constant light, followed by three cycles of 20 seconds of diffuse luminance flicker at 12.5 Hz and 80 seconds of constant light. Repetition mode was used for all follow-up tests to ensure that the same vessel segments were studied. If repetition mode was unable to automatically re-identify the vessel segments, the same segments were manually re-selected using an image of the previous examination location. Baseline vessel diameters were reported in MU, where 1 MU is equivalent to 1 µm for the Gullstrand eye. Maximum vessel dilation was calculated from the mean of the three measurement cycles as the maximum percentage increase in vessel diameter relative to baseline during 20 seconds of flicker stimulation.

6.4.6 Extraction of plasma (n = 12) for fluconazole quantitation

Milli-Q Water (70 µL; EMD Millipore, Billerica, USA) was added to plasma (50 µL) in a 2 ml Eppendorf® tube. Ethyl acetate (100%) (600 µL) was added and the sample vortexed (one minute) and centrifuged (2,200 g, five minutes, room temperature [RT]). A 550 µL aliquot (upper polar phase) was transferred into a glass insert, dried in vacuo then re-dissolved in 40 µL ethyl acetate for subsequent fluconazole quantitation by gas chromatography-mass spectrometry (GC-MS).

6.4.7 Extraction of plasma (n = 12) for salicylic acid quantitation

Plasma (50 µL) was transferred into a 1.5 ml Eppendorf® tube. Cold (4°C) methanol (150 µL) was added, vortexed (one minute), placed on ice (10 minutes), then centrifuged (2,200 rpm, 10 minutes, RT) to precipitate protein. A 75 µL aliquot was transferred into a glass insert, dried in vacuo and placed in a snaplock bag with silica gel prior to derivatisation by GC-MS. For derivatisation, plasma samples were treated for 30 minutes with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (40 µL) with mixing at 500 rpm at 37°C and allowed to rest for 60 minutes before injection into the GC-MS.

6.4.8 GC-MS

Samples (1 µL) were injected into a GC-MS comprised of a Gerstel 2.5.2 Autosampler (Gerstel GmbH, Mülheim, Germany), a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent Technologies, Santa Clara, USA). The MS was adjusted according to the manufacturer’s recommendations using tris-
(perfluorobutyl)-amine (CF43). GC was performed on a 30 m VF-5MS column with 0.2 μm film thickness and a 10 meter Integra guard column (Agilent Technologies, Santa Clara, USA).

Temperatures were set at 250°C for the injection inlet, 280°C for the MS transfer line, 250°C for the ion source and 150°C for the quadrupole. The carrier gas (helium) flow rate was 0.8 ml/min. (fluconazole) or 1.0 ml/min. (salicylic acid). Sample analysis was under the following temperature program: start injection at 50°C, hold for one minute, ramp temperature by 15°C/min. (fluconazole) or 25°C/min. (salicylic acid) to 325°C and heat two minutes (fluconazole) or three minutes (salicylic acid).

Retention times and mass spectra (unique qualifier ions) were identified and compared directly using commercially available standards of fluconazole (≥ 98% [HPLC], F8929-100MG, Sigma–Aldrich, Sydney, Australia) and salicylic acid (≥ 99% [BioXtra], SF922-100G, Sigma–Aldrich, Sydney, Australia). Drug concentrations were quantified from prepared calibration curves in the linear range: 10, 25, 50, 75, 100, 125, and 150 μM for fluconazole; and 10, 25, 50, 75 and 100 μM for salicylic acid.

Mass spectra were recorded at two scans/sec. with an m/z 50-600 scanning range. Both chromatograms and mass spectra were evaluated using the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for GC-MS. All matching mass spectra were additionally verified by determination of the retention time with authentic fluconazole and salicylic acid standards.

6.4.9 PGE₂ metabolite plasma concentration

Plasma PGE₂ metabolite concentrations before and after flicker light-induced retinal vasodilation on no-drug visits were quantified on previously unthawed samples using the Prostaglandin E Metabolite EIA Kit (Cayman Chemical, Ann Arbor, MI) as per manufacturer’s instructions. Briefly, proteins were precipitated with acetone and samples derivatised (37°C) overnight then further purified after acidification by ethyl acetate extraction. The standard curve (in duplicate) was obtained from eight 1:1 serial dilutions of standard from 50 pg/ml to 0.39 pg/ml. Samples were assayed undiluted in triplicate. The plate was developed by addition of Ellman’s reagent, incubated for 90 minutes and absorbance 412 nm read. The intra-assay coefficient of variation was 7.5%.

6.4.10 Statistical analyses

The mean maximum relative dilations during flicker stimulation and the resting arteriole and venule diameters were compared within each study. Group means were
compared using ANOVA for continuous variables or Fisher’s exact test for categorical variables. Pairwise comparisons with control or baseline groups used Student’s t-tests with Dunnett’s adjustments for multiple comparisons. Data were analysed in STATA (version 12.1; StataCorp LP, College Station, TX). $P < 0.05$ was considered significant. Based on our previous data, a crossover study with 12 participants would have 94% power to detect a 0.8% absolute ($\approx 20\%$ relative) reduction in arteriolar dilations one hour after the test-drug, assuming a within-subject SD of 0.5% and two-sided significance level of 0.05.

6.5 Results

The participants’ characteristics are described in Table 7. In total, 20 unique participants were recruited for the crossover and repeated measures studies. One participant completed all three studies, one completed the crossover and fluconazole repeated measures studies and another one completed the fluconazole and aspirin repeated measures studies. Different studies were separated by at least four weeks in participants who completed two or more studies.

In general, participants were young, mostly male and Caucasian with normal blood pressure, BMI and IOP. The proportion of Caucasians and blood pressure levels were lower in the crossover study compared with the fluconazole and aspirin repeated measures studies.

6.5.1 Crossover study

The maximum relative dilations of arteriole and venule segments are shown in Figure 14A. Drug treatments had no significant effect on arteriolar or venular dilations. No significant sequence, period or carryover effects were identified for the crossover study ($P > 0.05$ for all).

The resting diameters of arteriole and venule segments prior to flicker stimulation are shown in Figure 14B. Only venule diameters were significantly different between drug treatments ($P = 0.03$ by ANOVA), being driven by fluconazole, which reduced the mean $\pm 95\%$ CI resting venule diameters by $5 \pm 4$ MU ($P = 0.02$ after Dunnett’s adjustment). Similar to dilations, no significant effect on diameters was identified for test periods or the sequence of drugs and no significant carryover effects were found ($P > 0.05$ for all).

Mean (SD) plasma fluconazole and salicylate levels one hour post-ingestion of 400 mg oral fluconazole and 600 mg dispersible aspirin were 6.75 (2.62) $\mu g/ml$ and
13.7 (11.9) µg/ml, respectively. At control visits, mean (SD) plasma prostaglandin E₂ metabolite levels were 15.10 (17.08) pg/ml and 16.03 (19.33) pg/ml immediately before and after DVA tests, respectively. This corresponded to a mean (SD) difference in concentrations of 0.93 (3.37) pg/ml (P > 0.05).

Changes in retinal vasodilations during flicker stimulation and resting vessel diameters between control and drug visits are presented in Table 8, stratified by tertiles of plasma drug concentrations. No significant differences between tertiles of plasma drug concentrations were identified for arteriole or venule dilations during flicker stimulation or resting diameters (P > 0.05 for all).

6.5.2 Repeated measures studies

**Fluconazole**

The effects of fluconazole on flicker light-induced retinal vasodilation and resting arteriole and venule diameters are shown in Figure 15. Maximum vessel dilations were unchanged from baseline by fluconazole at any time point (P > 0.05 for all). Fluconazole did not significantly affect arteriole diameters over two hours, although the mean ± 95% CI of resting arteriole diameters was reduced by 3 ± 3 MU at 120 minutes after ingestion. In contrast, fluconazole significantly affected venule diameters over two hours (P < 0.001). Fluconazole reduced the mean ± 95% CI resting venule diameters by 3 ± 2 MU, 5 ± 2 MU and 6 ± 2 MU at 60, 90 and 120 minutes after fluconazole, respectively.

**Aspirin**

The effects of aspirin on flicker light-induced retinal vasodilation and resting arteriole and venule diameters are shown in Figure 16. Aspirin had no significant effect on the maximum dilations or resting diameters of arterioles or venules (P > 0.05 for all).

6.6 Discussion

Neither fluconazole nor aspirin reduced flicker light-induced retinal vasodilation in humans *in vivo*. This was a consistent finding both in our crossover and repeated measures studies. Indeed, our repeated measures studies appeared to suggest that fluconazole may actually increase, rather than decrease, dilations during flicker stimulation. However, in further analyses (not shown), fluconazole had no effect on maximum absolute vessel diameters and the apparent increase in relative dilations appeared to be a result of smaller diameters prior to flicker stimulation. Given that
fluconazole and aspirin inhibit the enzymes CYP2C and COX, respectively, our findings suggest that these EETs and PGs are not major contributors to retinal hyperaemia during flicker light stimulation in humans.

Our results contrast with recent rodent data that indicate these responses are a result of glial-derived EETs and PGs. This discrepancy may be due in part to the experimental models. These rodent studies used *ex vivo* retinas that had been surgically removed and perfused by oxygen bubbled through saline, not blood. Furthermore, the arterioles were pre-constricted with a thromboxane analogue. *In vivo* studies with cats and humans have consistently implicated nitric oxide as a major contributor to retinal hyperaemia during flicker light stimulation. Our results suggest that EETs and PGs are not major contributors to retinal hyperaemia in humans, and are consistent with the paradigm that nitric oxide is the primary mediator of these responses.

We did not find a significant effect of fluconazole on resting retinal arteriole diameters in either study. Arterioles were probably slightly constricted at 120 minutes after fluconazole ingestion, but the change was small and our study was not powered to detect differences in this outcome. This is consistent with the previous finding of radial artery constriction with intra-arterial fluconazole and L-NMMA, but not with either drug alone. In contrast, fluconazole consistently and significantly induced venule constriction in both our crossover and repeated measures studies. The greatest change was seen at 120 minutes after ingestion, with a constriction of ~4% from baseline. This suggests that EETs might play a role in the regulation of retinal blood flow under physiological conditions via a venous dilatory effect. Aspirin ingestion had no effect on either retinal arteriole or venule diameters.

We measured plasma fluconazole and salicylate concentrations to confirm the effectiveness of our oral drug administration. Plasma fluconazole levels were almost identical to those previously reported at one hour post-ingestion of 400 mg. Surprisingly, plasma salicylate levels were only 13.7 µg/ml at one hour post-ingestion of 600 mg dispersible aspirin, roughly one third of what was expected. This may have been due in part to the participants’ non-fasting status or samples taken too early to capture peak salicylate concentrations. However, as an irreversible inhibitor, the duration of aspirin is dependent on the rate of COX turnover, which is roughly six hours in humans. Similar doses of aspirin have potentiated, reduced or had no effect on cutaneous hyperaemia during iontophoresis, but no study has
examined salicylate concentrations with functional responses, as we have. We also compared changes between control and drug visits in retinal vessel dilations during flicker stimulation and resting diameters by tertiles of measured drug concentrations. We did not find any differences between tertiles of drug concentrations, but with only four participants per tertile, our crossover study may have had limited power to detect such differences.

Given that PGE$_2$ is the main vasodilator prostaglandin of glia$^3$, we hypothesised that plasma PGE$_2$ metabolite levels may increase immediately following retinal flicker stimulation. Plasma PGE$_2$ metabolite concentrations were highly variable between participants, but remained stable before and after our tests. Our results did not implicate PGE$_2$ as a mediator of retinal functional hyperaemia, although this was not unexpected given that our samples were very diluted from the source and obtained far from the eye at the cubital fossa.

Strengths of our study included our use of pharmacological inhibitors to investigate directly the contribution of EETs and PGs to flicker light-induced retinal vasodilation in humans and our quantification of drug levels. Secondly, we approached our research question using two study designs. The crossover study minimised bias and the effects of potential confounders, while our repeated measures studies characterised acute drug effects over two hours. Both approaches were statistically efficient and enabled testing for within-person effects without requiring large participant numbers. Finally, our DVA system allowed us to obtain accurate real-time measurements of retinal vessel diameters. Although vasodilation is considered an indirect measure of hyperaemia, this technique is currently more reliable than other techniques, such as laser Doppler velocimetry$^{19,262}$.

A major limitation of *in vivo* functional studies in humans with systemic drugs is that we are restricted to drugs approved for human use. Unfortunately, many of the drugs used by Newman and colleagues$^{32,33}$ are not safe for human research. In addition, we cannot determine how completely fluconazole and aspirin inhibited their respective enzymes at these doses. Finally, we did not measure visual acuity or refractive errors in our participants. However, only four of our participants had myopia and we did not previously find any relationship between refractive error and retinal flicker responses in young people$^{262}$. We did not consider our sample sizes to be a major limitation as our studies were designed to detect large within-person effects, which should have been evident if EETs or PGs were major mediators.
Given our results, additional *in vivo* animal studies are required to confirm the importance of EETs, PGs and nitric oxide in retinal functional hyperaemia. In particular, studies are needed to identify the cellular source of flicker light-stimulated nitric oxide production. Recent epidemiological studies indicate that DR progression is associated with wider retinal vessels\(^{275}\). Considering that EETs appear to regulate retinal venule diameters, the relationship between wider retinal vessels and EETs or PGs merits study in diabetes.

Our data do not support a major role for EETs or PGs in flicker light-induced retinal vasodilation. Given previous findings\(^5-^7\), nitric oxide is probably the principal mediator of this response. EETs appear to regulate retinal venular diameters to a small degree *in vivo* and might therefore play a minor role in the physiological regulation of retinal blood flow at rest.

### 6.7 Acknowledgements

We thank Dr. Jing Xie for expert statistical advice and Dr. Andrzej Januszewski (NHMRC Clinical Trials Centre, Sydney, Australia) for the PGE\(_2\) metabolite assay. Project funding was from the Royal Victorian Eye and Ear Hospital. The Centre for Eye Research Australia receives Operational Infrastructure Support from the Victorian Government. JN was supported by the Australian NHMRC Postgraduate Medical Scholarship (ID1038701). EL and GJD were supported by Australian NHMRC Research Fellowships (ID1045280 and 1003113).

### Table 6. Crossover study order of treatments (*n* = 12).

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<tr>
<td>A</td>
<td>Control</td>
<td>Fluconazole</td>
<td>Aspirin</td>
</tr>
<tr>
<td>B</td>
<td>Control</td>
<td>Aspirin</td>
<td>Fluconazole</td>
</tr>
<tr>
<td>C</td>
<td>Fluconazole</td>
<td>Control</td>
<td>Aspirin</td>
</tr>
<tr>
<td>D</td>
<td>Fluconazole</td>
<td>Aspirin</td>
<td>Control</td>
</tr>
<tr>
<td>E</td>
<td>Aspirin</td>
<td>Control</td>
<td>Fluconazole</td>
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<tr>
<td>F</td>
<td>Aspirin</td>
<td>Fluconazole</td>
<td>Control</td>
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Table 7. Baseline characteristics of participants.

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<td>Fluconazole</td>
</tr>
<tr>
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<td></td>
<td>Aspirin</td>
</tr>
<tr>
<td>Age, y</td>
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<tr>
<td>Ratio of men to women</td>
<td>13:7</td>
<td>9:3</td>
<td>4:2</td>
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<td>Ratio of Caucasians to other ethnicities</td>
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<td>7:5</td>
<td>6:0</td>
</tr>
<tr>
<td>Ratio of brown to other iris pigmentations</td>
<td>9:11</td>
<td>7:5</td>
<td>2:4</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>114 (11)</td>
<td>108 (8)</td>
<td>122 (8)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>74 (9)</td>
<td>70 (6)</td>
<td>79 (7)</td>
</tr>
<tr>
<td>Heart rate, beats/min.</td>
<td>64 (11)</td>
<td>62 (10)</td>
<td>64 (16)</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>24.9 (3.2)</td>
<td>24.6 (2.9)</td>
<td>25.0 (2.8)</td>
</tr>
<tr>
<td>IOP, mm Hg</td>
<td>13.8 (3.8)</td>
<td>12.5 (3.7)</td>
<td>15.2 (4.4)</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD) unless otherwise indicated. Participant characteristics were compared between studies by ANOVA for continuous variables or Fisher’s exact test for categorical variables.

Table 8. Changes in retinal vasodilations and resting calibres between control and drug visits by tertiles of plasma drug concentrations.

<table>
<thead>
<tr>
<th>Tertile</th>
<th>Range, µg/ml</th>
<th>Change vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arteriole Dilation, %</td>
<td>Venule Dilation, %</td>
</tr>
<tr>
<td>Fluconazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt; 5.0</td>
<td>0.0 (2.6)</td>
</tr>
<tr>
<td>2</td>
<td>5.0 to &lt; 7.8</td>
<td>-0.3 (1.6)</td>
</tr>
<tr>
<td>3</td>
<td>≥ 7.8</td>
<td>1.2 (1.6)</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.57</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&lt; 8.0</td>
<td>0.5 (1.8)</td>
</tr>
<tr>
<td>2</td>
<td>8.0 to &lt; 11.0</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>3</td>
<td>≥ 11.0</td>
<td>-1.6 (1.7)</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Data are expressed as means (SD). Results between tertiles of plasma drug concentrations were compared by ANOVA.
Figure 14. Crossover study. A: maximum retinal arteriole and venule dilations during flicker light stimulation. B: pre-flicker retinal arteriole and venule diameters. Data are expressed as means ± SEM. *P < 0.05 versus control.
Figure 15. Effects of fluconazole over two hours. A: maximum flicker light-induced retinal vasodilations. B: baseline retinal vessel diameters. Data are expressed as means ± SEM. * P < 0.05, *** P < 0.001 versus baseline.
Figure 16. Effects of aspirin over two hours. A: maximum flicker light-induced retinal vasodilations. B: baseline retinal vessel diameters. Data are expressed as means ± SEM.
CHAPTER 7. RETINAL NEURONAL AND VASCULAR
FUNCTION IN TYPE 1 DIABETES DURING EUGLYCAEMIC
AND HYPERGLYCAEMIC CLAMP
7.1 Context to the chapter

The first part of this chapter is presented in paper format, as for Chapters 4 to 6. This part is under review with Investigative Ophthalmology and Visual Science at the time of writing and has not yet been accepted for publication. It addresses the outcomes of Aim 4a, “To investigate the effects of euglycaemic and hyperglycaemic clamps on luminance-flicker induced retinal vasodilations, retinal ganglion cell function and systemic arterial elasticity in people with type 1 diabetes”, and Aim 4b, “To investigate the effect of antioxidant treatment prior to hyperglycaemic clamps on luminance flicker-induced retinal vasodilation, retinal ganglion cell function and systemic arterial elasticity in people with type 1 diabetes”, of my doctoral project.

Supplementary analyses, not submitted for publication, are presented in the latter part of this chapter: 1) additional data from systemic arterial elasticity and PERG studies; 2) within-subjects Pearson correlation coefficients with luminance flicker-induced retinal vasodilation responses; and 3) within-subjects Pearson correlation coefficients with other functional parameters.

The nature and proportion of my contributions and those of my collaborators to the work presented in this chapter are described in the Preface of this thesis.
RETINAL NEURONAL AND VASCULAR FUNCTION IN TYPE 1 DIABETES DURING EU GLYCEMIC AND HYPERGLYCEMIC CLAMP

Jonathan E. Noonan¹ BSc(Hons) MBBS; Glenn M. Ward²,³ DPhil FRACP; Marc Sarossy¹,⁴ MBBS FRANZCO; Ryan E.K. Man¹,⁵ BSc PhD; Thanh T. Nguyen¹ PhD FRANZCO; Alicia J. Jenkins⁶⁻⁸ MD FRACP; Gregory J. Dusting¹ PhD FBPharmcolS; Ecosse L. Lamoureux¹,⁵,⁹ MSc PhD.

1. Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, University of Melbourne, Melbourne, VIC, Australia
2. Departments of Endocrinology and Diabetes, and Clinical Biochemistry, St. Vincent’s Hospital, University of Melbourne, Melbourne, VIC, Australia
3. Department of Pathology, University of Melbourne, Melbourne, VIC, Australia
4. RMIT University, Melbourne, VIC, Australia
5. Singapore Eye Research Institute and Department of Ophthalmology, National University of Singapore, Singapore
6. Department of Medicine, St. Vincent’s Hospital, University of Melbourne, Melbourne, VIC, Australia
7. NHMRC Clinical Trials Centre, University of Sydney, Sydney, NSW, Australia
8. Queen’s University, Belfast, N. Ireland, United Kingdom
9. Duke-NUS Graduate Medical School, Singapore

Corresponding and senior author:
A/Prof Ecosse L. Lamoureux
Centre for Eye Research Australia
Department of Ophthalmology, University of Melbourne
32 Gibson Street, East Melbourne, Victoria 3002, Australia
Ph: +61 3 9929 8371
Fax: +61 3 9662-3859
Email: ecosse@unimelb.edu.au

Disclosures: Nothing to disclose.

Key words: oxidative stress, type 1 diabetes, hyperglycaemia, pattern electroretinogram, retinal blood flow.

Word count: Abstract 235; Manuscript (ex Abstract) 3,081.
7.2 Abstract

**Purpose:** To study changes in retinal neurovascular function and systemic vascular function between baseline, euglycaemia and hyperglycaemia in type 1 diabetes. A secondary aim was to study the effects of vitamin C on functional parameters during hyperglycaemia.

**Methods:** Twelve adults with type 1 diabetes participated in this crossover study. At both visits, a two-stage euglycaemic and hyperglycaemic clamp stabilised plasma glucose levels at 6 mmol/l and 15 mmol/l, respectively, during insulin infusions (6 pmol/kg/min.). Vitamin C (2 g) or placebo was given intravenously prior to hyperglycaemia. Radial artery pulsewave tonometry, pattern electroretinogram transient and steady-state responses and flicker-light induced retinal vasodilation were measured at baseline, euglycaemia and hyperglycaemia.

**Results:** Participants were (median [IQR]) aged 24 (20.5 – 30.0) years with 10.5 (3.8 – 17.0) years of diabetes. Euglycaemic clamp increased retinal venule maximum dilations and area under the curve during flicker by (mean [SD]) 1.7 (2.0) % and 28.7 (28.9) % x sec., respectively (both $P < 0.01$). Systemic arterial elasticity and ganglion cell function were stable across all conditions. Hyperglycaemia had no effect on flicker responses in the presence of high insulin levels. Similarly, no significant differences in functional parameters were observed between hyperglycaemia with vitamin C and placebo.

**Conclusions:** Euglycaemic clamping increases flicker-induced retinal venule dilation, while hyperglycaemia does not impair functional markers in the presence of high insulin. Thus, glucose control and insulin treatment improve retinal vascular function in type 1 diabetes.
7.3 Introduction

Diabetes mellitus affects approximately 8.3% of the world’s population. It’s main ocular complication, diabetic retinopathy (DR), affects roughly one third of people with diabetes and is a leading cause of adult-onset blindness. Grading of DR severity from colour fundus photos is the gold standard method to monitor the incidence and progression to vision-threatening DR. However, grading does not convey information about retinal neuronal and vascular function. Such markers could complement traditional grading to improve risk assessments for DR progression.

Flicker light-induced retinal vasodilation has attracted interest as a potential sensitive marker of retinal vascular function. This marker is impaired in people with diabetes, even in the absence of traditional DR signs. Hypothesised mechanisms of this response include ganglion cell activation and signalling via nitric oxide or arachidonic acid pathways. Ganglion cell function is disturbed in diabetes and this may contribute to reduced flicker-induced dilations. Correlations were found between flicker-induced retinal arteriole dilations and PERG N95 components in adults with and without diabetes. However, reduced flicker-induced vasodilation has also been observed in the absence of impaired PERG responses in adults with type 1 diabetes. These findings suggest that neuronal and vascular function in the retina are interrelated; and may provide useful information about retinal health in diabetes.

To this point it has been unclear whether retinal function in diabetes acutely fluctuates with glycaemia. Periods of hyperglycaemia, as are common in type 1 diabetes, are directly linked to endothelial dysfunction and systemic arterial stiffening and could similarly interfere with retinal function. One study found that flicker-induced venule dilations were reduced by hyperglycaemia and somatostatin-induced insulin suppression in non-diabetic people. This link has not previously been investigated in type 1 diabetes, where somatostatin would not be required. Normalisation of glucose levels with insulin restores retinal blood flow to normal levels in people with type 1 diabetes. Similar results may be expected for flicker-induced vasodilation. Ganglion cell function may be less likely to fluctuate with glucose control, although no study has formally reported on this relationship.

Hyperglycaemia is hypothesised to contribute to diabetic complications via the generation of oxidative stress. Antioxidants might therefore protect against DR, as was found for forms of age-related macular degeneration. Vitamin C (ascorbic acid)
preserves systemic arterial elasticity\textsuperscript{37} and endothelial function\textsuperscript{39, 40} during acute hyperglycaemia. Whether it could similarly protect vascular function in the retina is unknown.

We hypothesised that flicker light-induced retinal vasodilation would increase after normalisation of glucose levels and be reduced by hyperglycaemia in people with type 1 diabetes. We simultaneously studied ganglion cell function with the PERG and hypothesised that these responses would be stable across glucose conditions. We also studied systemic arterial elasticity to corroborate previous studies\textsuperscript{37, 38}. On alternate days, we gave vitamin C or placebo intravenously prior to hyperglycaemia to test whether vitamin C could prevent hyperglycaemia-induced retinal vascular dysfunction.

7.4 Methods

7.4.1 Participants

We recruited 12 people with type 1 diabetes from the University of Melbourne, the diabetes clinic at St. Vincent’s Hospital, Melbourne and through notices with Diabetes Australia (Victoria) and the Juvenile Diabetes Research Foundation Australia. Inclusion criteria were age $\geq$ 18 years and a self-reported history of type 1 diabetes with insulin treatment. Exclusion criteria were any major eye pathology other than refractive error, systemic conditions other than type 1 diabetes, epilepsy, oral hypoglycaemic medications, tropicamide sensitivity, smoking, pregnancy or lactation. In those with diabetes for more than five years, an eye check in the past year with no findings of DR was required. However, participants found to have signs of DR on formal grading after successful study completion were not excluded. All participants had their age, sex, BMI, BCVA, refractive error (spherical equivalent) and IOP recorded. BCVA was measured with a Logarithm of the Minimum Angle of Resolution (LogMAR) chart. IOP was measured after topical instillation of oxybuprocaine hydrochloride, 0.4%, and fluorescein sodium with a slitlamp-mounted Goldmann applanation tonometer (Haag-Streit, Bern, Switzerland). The study protocol followed the tenants of the Declaration of Helsinki and was approved by the St. Vincent’s Hospital Melbourne Human Research Ethics Committee D (096/13). Written informed consent was obtained from all participants after explanation of the nature and potential consequences of the study.

7.4.2 Study design

Participants were studied on two mornings separated by at least two weeks. Vitamin C (2 g in 100 ml sodium chloride 0.9%; Biological Therapies, Braeside, VIC,
Australia) was given intravenously at one visit and placebo (100 ml sodium chloride 0.9%) was given at the other visit immediately prior to the initiation of hyperglycaemia. Computer-assisted randomisation was used to assign six participants to vitamin C then placebo; and six participants to placebo then vitamin C. Both investigators and participants were masked to these treatments. We asked participants to cease any vitamin and herbal supplements from two weeks before their first visit until after their second visit. Participants were requested to fast from midnight the night before and were given individualised instructions for managing their insulin.

Tests of systemic arterial elasticity, retinal ganglion cell function and luminance flicker-induced retinal vasodilation were performed in order at baseline; after one hour of insulin and 30 minutes of euglycaemia; and a third time after 30 minutes of hyperglycaemia. Details of the glucose clamp techniques are described below. Each test took approximately 10 minutes to complete, including setup. Insulin and glucose infusions continued throughout euglycaemic and hyperglycaemic tests. Topical tropicamide 1% (Bausch & Lomb, Macquarie Park, NSW, Australia) was instilled in the right eye prior to the first and second group of functional tests above to permit the assessment of luminance flicker-induced retinal vasodilation. Baseline urine and blood samples were collected prior to insulin infusions and sent to St. Vincent’s Pathology (Melbourne, VIC, Australia) for measurement of HbA1c, serum creatinine and the urine albumin:creatinine ratio.

7.4.3 Glucose clamp

We used a modified two-stage euglycaemic and hyperglycaemic clamp based on the technique of DeFronzo and colleagues\textsuperscript{250}. After baseline tests, one 20 gauge intravenous cannula was inserted into the antecubital vein of each arm. One cannula was used for drug infusions and the other was for blood samples. Insulin (Actrapid, Novo Nordisk Pharmaceuticals, Baulkham Hills, NSW, Australia) was infused at a constant rate of 6 pmol/kg/min. Glucose 25% was infused at a rate necessary for the euglycaemic glucose level (6 ± 1 mmol/l). An iterative computer program was used to guide glucose infusion rates\textsuperscript{251}. Whole blood glucose levels were measured every 10 minutes with the YSI STAT Plus (YSI Incorporated, Yellow Springs, OH, USA) and glucose infusion rates were adjusted accordingly. If the initial glucose level was ≥ 10 mmol/l before the start of the insulin infusion, an initial individualised correction dose was given over 10 minutes to reduce the glucose level to 6 mmol/l within 30 minutes. Functional tests were repeated after a minimum of one hour of insulin and 30 minutes
of euglycaemia. Vitamin C or placebo was then infused over 10 minutes and the glucose infusion rate was increased to raise the glucose level to 15 mmol/l over approximately 30 minutes. After this level of hyperglycaemia was achieved, the infusion rate was reduced to maintain glucose levels in the hyperglycaemic range (15 ± 1 mmol/l). Functional tests were repeated a third time after 30 minutes of hyperglycaemia. Insulin and glucose infusions were then ceased. Participants were provided with a meal at the end of each visit and were observed for 45 minutes.

7.4.4 Systemic arterial elasticity

The HD/Pulsewave CR-2000 (Hypertension Diagnostics, Eagan, MN, USA) was used to assess arterial elasticity. Participants were positioned in a supine position for five minutes prior to measurements. A non-invasive blood pressure cuff was applied to the left arm while the right wrist was splinted to reduce movement and stabilise the radial artery. A pressure tonometer was positioned over the right radial artery and adjusted to the point of highest signal strength relative to the blood pressure. Arterial waveforms were recorded for 30 seconds and the diastolic portion was digitised at a sample rate of 200 Hz. Pressure waveforms were automatically fitted to a modified Windkessel model to determine the LAE (C1) and SAE (C2) artery elasticity as previously described.

7.4.5 Electrophysiology

PERG studies were conducted on the left eye only according to the ISCEV standard. Participants wore their own corrective lenses if required. Retinal potentials were recorded with DTL fibre electrodes positioned along the lower eyelid margin. Gold cup skin electrodes were positioned over the lateral canthus and central forehead as reference and ground electrodes, respectively. Electrode impedances were tested and were deemed acceptable if less than 5 kΩ. Signals were amplified by the Espion E3 Electoretinography System (Diagnosys LLC, Lowell, CA, USA) with a sampling rate of 1.2 kHz and band-pass filter of 0.625 to 100 Hz at a digital resolution of 32 bits. Potentials greater than ±50 µV were rejected as artefacts.

Participants were seated 57 cm from a 21-inch Cornerstone p1650 cathode ray tube monitor (Cornerstone, Fremont, CA, USA). The monitor was connected to a Pattern Stimulus Generator (Diagnosys LLC, Lowell, MA, USA) with a frame rate of 100 Hz. Stimuli were stationary black and white checkerboards with a size of 0.8°, mean luminance of 54 cd/m², and contrast of 100%. Transient responses were recorded.
from 150 sweeps of 200 milliseconds with a reversal rate of 1 Hz. Steady-state responses were recorded from 50 sweeps of one second with a reversal rate of 8.33 Hz.

We measured the amplitudes of the transient response P50 and N95 components. Next, the second harmonic (i.e. 16.67 Hz; 2F) of the steady-state response was assessed from eight cycles of 120 milliseconds. Data were exported to STATA version 12.1 (StataCorp LP, College Station, TX, USA) and the mean was subtracted from the signal. The discrete Fourier transform was used to obtain the 2F amplitude.

7.4.6 Luminance flicker-induced retinal vasodilation

Retinal arteriole and venule dilation during flicker stimulation was measured in the dilated right eye with the DVA (IMEDOS, Jena, Germany). Tests were conducted under normal ambient lighting (500 lux) for participant safety. Although this yields slightly smaller dilations than if tests are done in a darkened room\(^{277}\), the same lighting conditions were used for each study. Participants were seated and fixated on the tip of a fixation bar within the Zeiss FF450plus mydriatic camera (Carl Zeiss AG, Jena, Germany). The fundus was examined with a viewing angle of 30° under green light with an average luminance of 130 cd/m\(^2\). A high-contrast region of the fundus was selected as a target for the eye-tracking module of the DVA software. We then selected a straight segment of an arteriole and venule located approximately one half to two disc diameters from the optic disc margin. We preferred segments located in the superior temporal retina and at least one diameter from any bifurcation or neighbouring vessels. The same vessel segments were used for repeated tests.

Vessel diameters were recorded by the DVA software in measurement units (MU), where 1 MU is roughly equivalent to 1 µm\(^2\). Diameters were measured at 1 MU intervals along the length of each segment with a sampling rate of 25 Hz. Each test took 350 seconds to complete. This consisted of 50 seconds of constant light, followed by three cycles of 20 seconds of diffuse luminance flicker at 12.5 Hz and 80 seconds of constant light.

Vessel data were exported to STATA version 12.1 (StataCorp LP, College Station, TX, USA). Three periods of 100 seconds were defined per test. Each period consisted of the 30 seconds pre-flicker, 20 seconds of flicker and 50 seconds post-flicker. The periods were superimposed and locally weighted scatterplot smoothing with a bandwidth of 2% was used to smooth vessel temporal responses. Linear interpolation was used for sample intervals with missing data. Vessel dilations during flicker were standardised relative to the mean diameter over the 30 seconds prior to flicker. Pre-
flicker diameters (MU), maximum dilations (%) and the AUC during flicker (% x sec.) were extracted for each vessel.

7.4.7 Grading of retinopathy

Two-field colour fundus photography was done for both eyes at the conclusion of the first or second study visit. Images were centred on the macula and optic disc and taken with a Canon CR-2 digital non-mydriatic camera (Canon, Melville, NY, USA) with a viewing angle of 45°. DR was assessed by independent graders at the Centre for Eye Research Australia according to a modification of the Early Treatment Diabetic Retinopathy Study severity scale.176

7.4.8 Statistical analysis

Group means were compared across glucose conditions by ANOVA. Post-hoc comparisons for significant differences used Student’s t-tests with Sidak’s adjustments for unbalanced glucose factor levels. Data were analysed in STATA. Two-tailed \( P < 0.05 \) was considered significant. We estimated that our sample size of 12 would have a power of approximately 88% to detect a 1% difference in luminance flicker-induced retinal venule dilation between vitamin C and placebo treatments231, assuming from our previous data276 a SD of the difference of less than 1%.

7.5 Results

The participants’ baseline characteristics are described in Table 9. They were generally young with a normal or slightly increased BMI, and an even number of males and females. No participant had any evidence of renal complications. Three participants had signs of mild non-proliferative DR in at least one eye on fundus photography, despite an eye assessment in the prior year having found no DR. BCVA was excellent in all participants, regardless of DR status.

Mean study outcomes for each glucose condition are described in Table 10. As expected, plasma glucose levels were significantly reduced from baseline to euglycaemia and increased from euglycaemia to both hyperglycaemia arms. Free insulin levels significantly increased from baseline to euglycaemia and blood pressures tended to decrease after baseline.

Systemic arterial elasticity and ganglion cell function were unchanged between glucose conditions. Mean flicker responses are illustrated in Figure 17. Venule maximum dilations and AUC during flicker were mean (SD) 1.7 (2.0) % and 28.7 (28.9) % x sec. larger, respectively, after euglycaemic clamp. These parameters were
not significantly different between euglycaemia and either hyperglycaemia arm. Arteriole maximum dilations and AUC during flicker appeared slightly higher under hyperglycaemia with vitamin C but significant differences were not found ($P = 0.07$ and $P = 0.09$ by ANOVA, respectively). Pre-flicker vessel diameters were unchanged between glucose conditions.

Other than plasma glucose levels, no factor changed significantly from euglycaemia to either hyperglycaemia arm. Similarly, no significant differences were found between vitamin C and placebo at hyperglycaemia. Systolic and diastolic blood pressures were 4 mmHg ($P = 0.02$) and 3 mmHg ($P = 0.04$) lower at second visits. SAE was 1.2 ml/mmHg x 100 higher at second visits. No carryover effects were identified for any factor.

7.6 Discussion

Euglycaemic clamp improves luminance flicker-induced retinal venule dilation in adults with type 1 diabetes. This improvement persisted after hyperglycaemia in the presence of high insulin levels, which suggests that this effect was predominantly mediated by insulin. PERG parameters were stable across glucose conditions. The stability of these parameters implies that ganglion cell dysfunction in diabetes probably occurs gradually without fluctuations in parallel with glucose levels\textsuperscript{214-218}. Our findings are similar to those of Pemp and colleagues\textsuperscript{234}, who found that people with type 1 diabetes had increased retinal blood flow at baseline that reduced to non-diabetic levels during euglycaemic clamp. Our data support the notion that glucose control with insulin in type 1 diabetes improves retinal vascular function.

Contrary to our hypothesis, we did not find any effect of hyperglycaemia on retinal or systemic vascular parameters. Dorner and colleagues\textsuperscript{231} found that flicker-induced venule dilations were reduced by approximately 1% after 30 minutes of hyperglycaemia with a target blood glucose level of 16.6 mmol/l. Arteriole dilations were also smaller but not significantly different from baseline. Two other studies found rapid systemic arterial stiffening during acute hyperglycaemia in non-diabetic and type 1 diabetic people\textsuperscript{37,38}, Unlike the three studies mentioned above\textsuperscript{37,38,231}, we used insulin infusions during hyperglycaemia to standardise comparisons between glucose conditions. High insulin levels protected rodents from oxidative stress during acute hyperglycaemia\textsuperscript{278}. Furthermore, euglycaemic clamp reduced oxidative stress in people
with type 2 diabetes\textsuperscript{279}. Thus, high insulin levels appear to be protective against hyperglycaemia-induced oxidative stress and vascular dysfunction.

It is important to discuss the haemodynamic effects of insulin and glucose. Insulin and glucose generally cause vasodilation\textsuperscript{232, 233}, although insulin can reduce retinal blood flow in people with diabetes\textsuperscript{234, 235}. Consistent with these effects, we observed small blood pressure reductions after the start of insulin infusions. Pre-flicker retinal vessel diameters were unchanged between glucose conditions. This may have been due to the opposing effects of insulin and glucose-lowering on the retinal circulation. Small reductions in blood pressure and small artery stiffness were noted at second visits, likely due to lower participant stress.

Unlike Mullan and colleagues\textsuperscript{37}, we were unable to demonstrate a significant benefit of vitamin C treatment on retinal or systemic vascular function. The main reason for this was a lack of vascular dysfunction during hyperglycaemia, which as discussed above was probably due to the protective effects of high insulin levels. Although it did not reach statistical significance, our data suggest that vitamin C may improve flicker-induced retinal arteriole dilation even in the presence of high insulin and glucose levels. However, this was unexpected and our study was probably underpowered to detect this effect.

A challenge in our study was the length of time to study each participant. We saw each participant twice and each visit took at least four hours to complete. The elimination of insulin infusions in future studies should simplify procedures and improve participant convenience. A once-off insulin dose would be necessary for type 1 diabetes participants with higher “fasting” blood glucose levels. Our sample size of 12 was small but reasonable, given our pre-study assumptions and comparisons to similarly designed studies\textsuperscript{7, 37, 231, 276}. The main strength of our study was our focus on within-subject effects, as the variability of functional parameters in the same person is much less than the variability between different individuals. This reduces the need for large sample sizes as is often seen in parallel group studies.

In conclusion, our study demonstrates that retinal venule dilation during flicker stimulation robustly improves with euglycaemic clamp in type 1 diabetes. The absence of hyperglycaemic effects during insulin infusions suggests that insulin improves retinal blood flow regulation and this is mediated by more than simple glucose-lowering. Retinal ganglion cell function is generally stable in the short-term. Antioxidants such as
vitamin C may protect retinal vascular function in type 1 diabetes, but more evidence is required to study this hypothesis.

7.7 Acknowledgements

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Table 9. Participants’ characteristics at baseline ($n = 12$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>24 (20.5 – 30)</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>6/6</td>
</tr>
<tr>
<td>Diabetes duration, y</td>
<td>10.5 (3.8 – 17)</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>24.2 (21.4 – 25.8)</td>
</tr>
<tr>
<td>IOP, mmHg*</td>
<td>14 (13 – 16)</td>
</tr>
<tr>
<td>BCVA, LogMAR chart*</td>
<td>-0.05 (-0.1 – 0.0)</td>
</tr>
<tr>
<td>Refractive error, spherical equivalent*</td>
<td>-1.25 (-2 – -0.25)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>8.1 (7.1 – 8.3)</td>
</tr>
<tr>
<td>Serum creatinine, µmol/l</td>
<td>73.5 (61.5 – 77)</td>
</tr>
<tr>
<td>Urine albumin:creatinine ratio</td>
<td>0.3 (0.2 – 0.5)</td>
</tr>
<tr>
<td>DR status†</td>
<td></td>
</tr>
<tr>
<td>No DR</td>
<td>7</td>
</tr>
<tr>
<td>MA only</td>
<td>2</td>
</tr>
<tr>
<td>Mild NPDR</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are medians (IQR) except where indicated. *Mean from both eyes. †Status of the worse eye. MA, microaneurysms; NPDR, non-proliferative DR.
Table 10. Participant factors grouped by glucose conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Euglycaemia (6 mmol/l)</th>
<th>Vitamin C</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/l</td>
<td>8.4 (3.6)</td>
<td>6.1 (0.8)**</td>
<td>15.0 (1.9)**</td>
<td>14.4 (0.8)**</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>65 (24)</td>
<td>334 (63)**</td>
<td>330 (58)**</td>
<td>338 (75)**</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>120 (11)</td>
<td>115 (7)</td>
<td>113 (10)*</td>
<td>112 (9)*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>66 (7)</td>
<td>62 (3)</td>
<td>60 (5)*</td>
<td>61 (5)*</td>
</tr>
<tr>
<td>Heart rate, beats/min.</td>
<td>66 (8)</td>
<td>64 (7)</td>
<td>65 (9)</td>
<td>63 (7)</td>
</tr>
<tr>
<td><strong>Systemic arterial elasticity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAE, ml/mmHg x 10</td>
<td>17.4 (4.0)</td>
<td>17.6 (4.3)</td>
<td>17.1 (3.1)</td>
<td>18.1 (4.7)</td>
</tr>
<tr>
<td>SAE, ml/mmHg x 100</td>
<td>10.3 (1.7)</td>
<td>10.5 (2.1)</td>
<td>11.3 (2.8)</td>
<td>11.1 (1.7)</td>
</tr>
<tr>
<td><strong>Pattern electroretinogram</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P50 amplitude, µV</td>
<td>4.8 (1.4)</td>
<td>4.4 (1.1)</td>
<td>5.0 (1.2)</td>
<td>4.8 (1.5)</td>
</tr>
<tr>
<td>N95 amplitude, µV</td>
<td>-6.4 (1.6)</td>
<td>-6.4 (1.8)</td>
<td>-6.4 (1.3)</td>
<td>-6.7 (2.0)</td>
</tr>
<tr>
<td>2F amplitude, µV</td>
<td>1.4 (0.5)</td>
<td>1.3 (0.4)</td>
<td>1.6 (0.3)</td>
<td>1.4 (0.5)</td>
</tr>
<tr>
<td><strong>Retinal arterioles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-flicker diameter, MU</td>
<td>125 (19)</td>
<td>125 (21)</td>
<td>122 (19)</td>
<td>123 (21)</td>
</tr>
<tr>
<td>Max dilation, %</td>
<td>3.2 (2.4)</td>
<td>3.6 (2.4)</td>
<td>4.4 (3.5)</td>
<td>3.7 (2.3)</td>
</tr>
<tr>
<td>AUC, % x sec.</td>
<td>31.2 (32.3)</td>
<td>35.8 (32.1)</td>
<td>45.5 (45.3)</td>
<td>39.9 (35.8)</td>
</tr>
<tr>
<td><strong>Retinal venules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-flicker diameter, MU</td>
<td>143 (19)</td>
<td>142 (19)</td>
<td>141 (18)</td>
<td>140 (18)</td>
</tr>
<tr>
<td>Max dilation, %</td>
<td>3.3 (3.4)</td>
<td>5.0 (4.1)**</td>
<td>4.7 (2.8)</td>
<td>5.0 (3.7)*</td>
</tr>
<tr>
<td>AUC, % x sec.</td>
<td>15.9 (44.1)</td>
<td>44.6 (50.8)**</td>
<td>44.3 (35.0)**</td>
<td>43.9 (37.8)*</td>
</tr>
</tbody>
</table>

Data are means (SD). Baseline, euglycaemia n = 24; hyperglycaemia n = 12 per treatment. *P < 0.05, **P < 0.01, ***P < 0.001 vs. baseline.
Figure 17. Mean relative arteriole (A) and venule (B) dilations. Bars indicate flicker. Solid lines, baseline; long dashed lines, euglycaemia; dotted lines, hyperglycaemia (vitamin C); short dashed lines, hyperglycaemia (placebo).
7.8 Supplementary analyses

The following supplementary analyses were not presented in the manuscript submitted for publication in *Investigative Ophthalmology and Visual Science*.

7.8.1 Supplementary data from Study 4 across glucose conditions

Mean (SD) supplementary outcomes from Study 4 that were not presented for publication are presented in Table 11. **Supplementary data across glucose conditions.** SVR was unchanged between glucose conditions. The PERG transient response N35 amplitude and implicit time, P50 implicit time and N95 implicit time were unchanged between glucose conditions. Similarly, the PERG steady-state response 2F amplitude with 7° check sizes, 2F proportion with 0.8° check sizes and 2F proportion with 7° check sizes were unchanged between glucose conditions. However, the ratio of the 0.8° check size 2F amplitude to the 7° check size 2F amplitude was significantly increased after hyperglycaemia with vitamin C compared to baseline \((P = 0.02\) after Sidak’s adjustment). No statistically significant differences were observed between euglycaemia and either hyperglycaemia arm or between the vitamin C and placebo hyperglycaemia arms.

7.8.2 Correlations with luminance flicker-induced arteriole and venule dilation

Within-subjects Pearson’s correlation coefficients with dilation responses are presented in Table 12. The arteriole AUC during flicker was positively correlated with plasma glucose levels \((r = 0.27, P = 0.04)\). Arteriole and venule maximum dilations and AUC during flicker were all positively correlated with free insulin levels. In addition, arteriole maximum dilations \((r = 0.26, P = 0.04)\) and AUC during flicker \((r = 0.26, P = 0.04)\) were positively correlated with LAE. No significant within-subjects correlations were identified between dilation responses and ganglion cell functional parameters.

I performed sensitivity analysis without data from hyperglycaemia with vitamin C in case this confounded correlations with arteriole and venule dilations. These data are presented in Table 13. With data after vitamin C excluded, glucose was no longer significantly correlated with arteriole AUC during flicker and insulin was no longer significantly correlated with arteriole maximum dilation or AUC during flicker. Insulin remained significantly positively correlated with venule maximum dilation and AUC during flicker. LAE also remained significantly positively correlated with arteriole maximum dilation and AUC during flicker.
7.8.3 Correlations with other parameters

Within-subjects Pearson’s correlation coefficients with LAE and SAE are presented in Table 14. Systolic blood pressure was significantly negatively correlated with both LAE and SAE. Expressed differently, reduced systemic arterial elasticity parameters were correlated with increased systolic blood pressures. Neither LAE nor SAE were significantly correlated with PERG ganglion cell amplitudes.

Within-subjects Pearson’s correlation coefficients with PERG amplitudes are presented in Table 15. Insulin levels were borderline \( (P = 0.05) \) negatively correlated with 7° 2F amplitudes. Systolic blood pressure was significantly negatively correlated with 0.8° 2F amplitudes, whilst diastolic blood pressure was significantly positively correlated with N95 amplitudes (meaning that absolute N95 amplitudes were smaller with higher diastolic blood pressures).

Sensitivity analysis excluding data from hyperglycaemia with vitamin C is presented in Table 16 for LAE and SAE and in Table 17 for PERG amplitudes. With these data excluded, systolic blood pressure remained significantly negatively correlated with LAE and SAE. Correlations between insulin and 7° 2F amplitudes and between diastolic blood pressure and N95 amplitudes remained significant, but the correlation between systolic blood pressure and 0.8° 2F amplitudes was no longer significant.

### Table 11. Supplementary data across glucose conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Euglycaemia (6 mmol/l)</th>
<th>Vitamin C</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systemic arterial elasticity</strong></td>
<td>SVR, mmHg x min/l</td>
<td>14.5 (1.5)</td>
<td>14.3 (1.7)</td>
<td>13.9 (2.4)</td>
</tr>
<tr>
<td><strong>Pattern electroretinogram</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N35 amplitude, µV</td>
<td>-0.9 (0.6)</td>
<td>-0.5 (0.4)</td>
<td>-1.0 (1.1)</td>
<td>-0.8 (0.6)</td>
</tr>
<tr>
<td>N35 implicit time, ms.</td>
<td>27.0 (2.2)</td>
<td>28.2 (2.7)</td>
<td>27.3 (2.4)</td>
<td>26.3 (2.0)</td>
</tr>
<tr>
<td>P50 implicit time, ms.</td>
<td>49.7 (2.1)</td>
<td>49.8 (2.0)</td>
<td>49.8 (2.2)</td>
<td>48.9 (2.2)</td>
</tr>
<tr>
<td>N95 implicit time, ms.</td>
<td>103.3 (3.7)</td>
<td>104.1 (5.0)</td>
<td>104.6 (4.3)</td>
<td>103.2 (7.5)</td>
</tr>
<tr>
<td>0.8° 2F proportion, %</td>
<td>9.1 (3.5)</td>
<td>8.9 (2.1)</td>
<td>10.2 (2.7)</td>
<td>9.3 (3.4)</td>
</tr>
<tr>
<td>7° 2F amplitude, µV</td>
<td>1.3 (0.4)</td>
<td>1.1 (0.2)</td>
<td>1.2 (0.3)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td>7° 2F proportion, %</td>
<td>9.0 (2.9)</td>
<td>8.0 (2.3)</td>
<td>8.2 (3.2)</td>
<td>7.9 (2.7)</td>
</tr>
<tr>
<td>0.8°:7° amplitude ratio</td>
<td>1.1 (0.3)</td>
<td>1.2 (0.3)</td>
<td>1.4 (0.6)*</td>
<td>1.2 (0.3)</td>
</tr>
</tbody>
</table>

Data are means (SD). 2F; second harmonic frequency (16.67 Hz); SVR, systemic vascular resistance. \(*P < 0.05\) vs. baseline.
Table 12. Within-subjects Pearson’s correlation coefficients with DVA parameters (n = 72).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Arterioles</th>
<th>Venules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max dilation</td>
<td>AUC</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>0.22 (0.08)</td>
<td>0.27 (0.04)</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.26 (0.04)</td>
<td>0.27 (0.04)</td>
</tr>
<tr>
<td>Systolic pressure</td>
<td>-0.07 (0.57)</td>
<td>-0.12 (0.37)</td>
</tr>
<tr>
<td>Diastolic pressure</td>
<td>0.01 (0.94)</td>
<td>-0.02 (0.90)</td>
</tr>
<tr>
<td><strong>Systemic arterial elasticity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large artery elasticity</td>
<td>0.26 (0.04)</td>
<td>0.26 (0.04)</td>
</tr>
<tr>
<td>Small artery elasticity</td>
<td>0.14 (0.27)</td>
<td>0.16 (0.22)</td>
</tr>
<tr>
<td><strong>Pattern electroretinogram</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P50 amplitude</td>
<td>0.16 (0.23)</td>
<td>0.15 (0.24)</td>
</tr>
<tr>
<td>N95 amplitude</td>
<td>-0.07 (0.60)</td>
<td>-0.06 (0.67)</td>
</tr>
<tr>
<td>0.8° 2F amplitude</td>
<td>0.05 (0.69)</td>
<td>0.02 (0.87)</td>
</tr>
<tr>
<td>7° 2F amplitude</td>
<td>-0.03 (0.79)</td>
<td>-0.09 (0.47)</td>
</tr>
</tbody>
</table>

Data are expressed as Pearson’s within-subject r (P). 2F, second harmonic frequency (16.67 Hz); AUC, area under the curve.

Table 13. Sensitivity analysis for within-subjects Pearson’s correlation coefficients excluding data from hyperglycaemia with vitamin C (n = 60).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Arterioles</th>
<th>Venules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max dilation</td>
<td>AUC</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>0.15 (0.31)</td>
<td>0.23 (0.11)</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.22 (0.12)</td>
<td>0.24 (0.10)</td>
</tr>
<tr>
<td>Systolic pressure</td>
<td>-0.01 (0.94)</td>
<td>-0.06 (0.68)</td>
</tr>
<tr>
<td>Diastolic pressure</td>
<td>0.05 (0.74)</td>
<td>0.04 (0.79)</td>
</tr>
<tr>
<td><strong>Systemic arterial elasticity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large artery elasticity</td>
<td>0.29 (0.04)</td>
<td>0.34 (0.02)</td>
</tr>
<tr>
<td>Small artery elasticity</td>
<td>0.12 (0.41)</td>
<td>0.17 (0.24)</td>
</tr>
<tr>
<td><strong>Pattern electroretinogram</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P50 amplitude</td>
<td>0.11 (0.44)</td>
<td>0.07 (0.61)</td>
</tr>
<tr>
<td>N95 amplitude</td>
<td>-0.10 (0.48)</td>
<td>-0.07 (0.63)</td>
</tr>
<tr>
<td>0.8° 2F amplitude</td>
<td>0.01 (0.96)</td>
<td>-0.03 (0.85)</td>
</tr>
<tr>
<td>7° 2F amplitude</td>
<td>-0.04 (0.78)</td>
<td>-0.11 (0.46)</td>
</tr>
</tbody>
</table>

Data are expressed as Pearson’s within-subject r (P). 2F, second harmonic frequency; AUC, area under the curve.
Table 14. Within-subjects Pearson’s correlation coefficients for systemic arterial elasticity parameters (n = 72).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Large artery elasticity</th>
<th>Small artery elasticity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose</td>
<td>0.00 (1.00)</td>
<td>0.15 (0.25)</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.04 (0.77)</td>
<td>0.16 (0.22)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>-0.32 (0.01)</td>
<td>-0.35 (0.006)</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.19 (0.13)</td>
<td>-0.21 (0.11)</td>
</tr>
</tbody>
</table>

**Pattern electroretinogram**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Large artery elasticity</th>
<th>Small artery elasticity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P50 amplitude</td>
<td>0.02 (0.88)</td>
<td>0.06 (0.67)</td>
</tr>
<tr>
<td>N95 amplitude</td>
<td>0.15 (0.25)</td>
<td>-0.10 (0.42)</td>
</tr>
<tr>
<td>0.8° 2F amplitude</td>
<td>0.19 (0.14)</td>
<td>0.22 (0.10)</td>
</tr>
<tr>
<td>7° 2F amplitude</td>
<td>0.00 (0.97)</td>
<td>0.00 (0.98)</td>
</tr>
</tbody>
</table>

Data are expressed as Pearson’s within-subject r (P). 2F, second harmonic frequency (16.67 Hz).

Table 15. Within-subjects Pearson’s correlation coefficients for pattern electroretinogram amplitudes (n = 72).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transient response</th>
<th>Steady-state response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P50 amplitude</td>
<td>N95 amplitude</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>0.11 (0.39)</td>
<td>-0.07 (0.60)</td>
</tr>
<tr>
<td>Insulin</td>
<td>-0.13 (0.34)</td>
<td>0.01 (0.93)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>blood</td>
<td>-0.19 (0.13)</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>blood</td>
<td>-0.18 (0.17)</td>
</tr>
</tbody>
</table>

Data are expressed as Pearson’s within-subject r (P). 2F, second harmonic frequency (16.67 Hz).
Table 16. Sensitivity analysis for within-subjects Pearson’s correlation coefficients for systemic arterial elasticity parameters excluding data from hyperglycaemia with vitamin C ($n = 60$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Large artery elasticity</th>
<th>Small artery elasticity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose</td>
<td>0.08 (0.60)</td>
<td>0.13 (0.38)</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.05 (0.73)</td>
<td>0.15 (0.31)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>-0.38 (0.007)</td>
<td>-0.31 (0.03)</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.18 (0.21)</td>
<td>-0.11 (0.44)</td>
</tr>
</tbody>
</table>

**Pattern electroretinogram**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Large artery elasticity</th>
<th>Small artery elasticity</th>
</tr>
</thead>
<tbody>
<tr>
<td>P50 amplitude</td>
<td>0.09 (0.53)</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>N95 amplitude</td>
<td>0.08 (0.58)</td>
<td>-0.15 (0.30)</td>
</tr>
<tr>
<td>0.8° 2F amplitude</td>
<td>0.23 (0.11)</td>
<td>0.16 (0.26)</td>
</tr>
<tr>
<td>7° 2F amplitude</td>
<td>0.16 (0.28)</td>
<td>0.05 (0.72)</td>
</tr>
</tbody>
</table>

Data are expressed as Pearson’s within-subject $r$ ($P$). 2F, second harmonic frequency (16.67 Hz).

Table 17. Sensitivity analysis for within-subjects Pearson’s correlation coefficients for pattern electroretinogram amplitudes excluding data from hyperglycaemia with vitamin C ($n = 60$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transient response</th>
<th>Steady-state response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P50 amplitude</td>
<td>N95 amplitude</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>0.02 (0.92)</td>
<td>-0.07 (0.61)</td>
</tr>
<tr>
<td>Insulin</td>
<td>-0.19 (0.19)</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>-0.16 (0.28)</td>
<td>0.18 (0.21)</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>-0.13 (0.38)</td>
<td>0.37 (0.008)</td>
</tr>
</tbody>
</table>

Data are expressed as Pearson’s within-subject $r$ ($P$). 2F, second harmonic frequency (16.67 Hz).
CHAPTER 8. DISCUSSION
8.1 Summary of key findings

This doctoral project investigated potential mechanisms of reduced luminance flicker-induced retinal vasodilation in people with and without type 1 diabetes. It consisted of four original research studies.

The primary aim of Study 1 was to investigate the impact of different test conditions on these responses. I explored the effect of repeated testing within five or 30 minutes on luminance flicker-induced retinal vasodilation and pre-flicker vessel diameters in healthy people. I found that luminance flicker-induced arteriole dilation was reduced when tests were repeated within five minutes. After 30 minutes, these arteriole responses recovered to be equivalent to the first test. Venule responses were slightly smaller in tests repeated within five minutes, but this change was not statistically different. Importantly, the pre-flicker diameters of arterioles and venules were unchanged between all three tests.

For Study 2, I studied the impact of reduced and normal ambient lighting conditions on luminance flicker-induced retinal vasodilation in healthy people. Echoing the results of my first study, I found that luminance flicker-induced arteriole dilation was reduced in tests conducted under higher ambient lighting. Venule dilations were slightly smaller with higher ambient lighting, but were not statistically significantly different. Pre-flicker arteriole and venule diameters were once again unaffected.

The objective of Study 3 was to investigate the potential role of the arachidonic acid metabolites, EETs and PGs, as mediators of luminance flicker-induced retinal vasodilation in healthy people. Using fluconazole and aspirin to inhibit EETs and PGs, respectively, I found no evidence to suggest that either drug inhibits luminance flicker-induced retinal vasodilation. In contrast to my original hypothesis, neither EETs nor PGs appeared to be important mediators of these responses in healthy humans. My only consistent finding was that fluconazole caused venule constriction, which may implicate EETs in the regulation of normal venular tone. These findings indirectly support the hypothesis that luminance flicker-induced retinal vasodilation is primarily mediated by nitric oxide.

My final study was designed to simultaneously address two main questions. First, I investigated changes in systemic arterial elasticity, retinal ganglion cell function and luminance flicker-induced retinal vasodilation between baseline, euglycaemia and hyperglycaemia in otherwise healthy adults with type 1 diabetes (Aim 4a). I simultaneously investigated whether vitamin C affects changes in these functional
parameters between euglycaemia and hyperglycaemia (Aim 4b). I found strong evidence that euglycaemic clamps improve luminance flicker-induced retinal venule dilation. Unexpectedly, acute hyperglycaemia did not reverse these improvements. Furthermore, I did not find any statistically significant differences in functional parameters between euglycaemia and hyperglycaemia or between hyperglycaemia with vitamin C and placebo.

In supplementary analyses for Study 4, I assessed within-subject Pearson’s correlation coefficients for maximum dilations and AUC during luminance flicker, PERG amplitudes and systemic arterial elasticity using the method described by Bland and Altman. Free insulin levels were positively correlated with arteriole and venule maximum dilations and AUC during flicker. Correlations were much stronger for venule dilations. Strong correlations between free insulin levels and venule dilation parameters are consistent with my observed improvements in luminance flicker-induced retinal venule dilation during euglycaemic clamps. Unexpectedly, plasma glucose levels were positively correlated with the arteriole AUC during flicker. My original hypothesis was that hyperglycaemia would impair arteriole flicker responses. This result was likely a result of half of my hyperglycaemic observations occurring after vitamin C treatment. Indeed, when vitamin C observations were excluded, arteriole responses were no longer correlated with plasma glucose or free insulin levels. Arteriole and venule flicker responses were not correlated with PERG amplitudes, which would reflect the short-term stability of ganglion cell function within each individual. In addition, arteriole maximum dilations and AUC during flicker were positively correlated with LAE. This suggests that more elastic retinal arterioles may permit more effective blood delivery during periods of increased neuronal activity. However, these supplementary analyses should be interpreted with caution, given the relatively small sample size.

8.2 Supplementary discussions

The purpose of this section is to discuss the pertinent findings of my doctoral project that have not been covered elsewhere in this thesis. Its purpose is not to reiterate detailed discussions that have already been presented as part of a paper. The main points from Studies 1 to 4 that have already been covered in paper format in Chapters 4 to 7, respectively, will not be repeated here.
8.2.1 Study 1: Retinal arteriolar dilation to flicker light is reduced on short-term re-testing

I found that arteriole dilation in response to 12.5 Hz diffuse green luminance flicker is reduced if tests are repeated with minimal recovery time between tests. This was important to establish for my subsequent studies that used repeated observations for each participant. If test conditions are difficult, such as if pupil dilation is sub-optimal, it may be necessary to stop and provide a short rest period before persisting with the test.

The most plausible explanation for smaller flicker-induced arteriole dilations on repeated testing is light adaptation to the mildly bright (130 cd/m$^2$) green light used throughout the test. Indeed, participants frequently reported seeing a purple or pink circle over their central vision immediately after the tests. This may correspond to desensitisation of the M-cones over the course of the tests$^{255, 256}$. This hypothesis of light adaptation is consistent with the findings of Riva and colleagues$^{88}$, who observed that a 30% increase in optic nerve blood flow could be produced with decreasing stimulus intensities during dark adaptation in anaesthetised cats.

The flicker stimulus used in this study, and throughout my doctoral project, was luminance flicker with green light, a temporal frequency of 12.5 Hz and a modulation depth of approximately 100% depending on the level of ambient lighting. As described in my literature review (Chapter 2), this type of flicker stimulus preferentially activates parasol (magnocellular) retinal ganglion cells$^{11}$. However, luminance flicker with a frequency of 12.5 Hz and high cone contrasts could also stimulate midget (parvocellular) ganglion cells$^{11}$. Both parasol and midget ganglion cells are coupled to M-cones: in parasol cells this connection is via diffuse bipolar cells, whereas M-cones are connected to midget cells via midget bipolar cells$^{58, 60, 68, 69}$. Thus, luminance flicker stimulation with green light at 12.5 Hz most likely produces retinal vasodilation via M-cone-driven parasol ganglion cell activity.

Alternative explanations for reduced luminance flicker-induced retinal arteriole dilations on repeat testing may include habituation of ganglion cells to luminance flicker, exhaustion of the signalling molecules that mediate vasodilation or reduced sensitivities of vascular smooth muscle to the signalling molecules. Significant ganglion cell habituation is unlikely to have occurred, given that each flicker period only lasted 20 seconds, only three flickers were delivered per test and each period was separated by at least 80 seconds. Minimal ganglion cell habituation would be expected over each 20
second flicker period\textsuperscript{280}. Furthermore, exhaustion of vasodilatory signalling molecules or desensitisation of vascular smooth muscle would be similarly unlikely, given that optic nerve blood flow appears to steadily increase over at least eight minutes of continuous 10 Hz flicker\textsuperscript{1}.

I did not find any statistically significant relationships between luminance flicker-induced arteriole or venule dilation and other participant factors. Studying the relationship between flicker responses and participant factors was not a pre-specified outcome and my study was probably underpowered to detect these relationships if they exist.

\textbf{8.2.2 Study 2: Flicker-induced retinal arteriole dilation is reduced by ambient lighting}

I recognised that it would not be feasible to conduct all my studies under reduced lighting, so I studied whether luminance flicker-induced arteriole or venule dilation would be affected by higher ambient lighting. Arteriole dilations were 0.7\% smaller under normal compared with reduced ambient lighting. I attributed this to an effective reduction in the modulation depth of luminance flicker at the retina. I estimated the modulation depth to be approximately 100\% under reduced ambient lighting, compared with 73\% under normal ambient lighting. My results are consistent with previous observations of larger increases in optic nerve blood flow with larger luminance flicker modulation depths\textsuperscript{90}.

Even though luminance flicker-induced retinal arteriole dilations are slightly smaller under normal compared with reduced ambient lighting, tests under normal ambient lighting may be preferred. Such tests would not induce dark adaptation in photoreceptors. In contrast to light adaptation, which as discussed under \textbf{Aim 1}, may decrease retinal vasodilations, dark adaptation may increase vasodilations to a given flicker stimulus\textsuperscript{1, 88}. In addition, tests conducted under normal ambient lighting may be technically easier because of better visibility for the examiner and test subject. Normal ambient lighting might therefore reduce the variability of test responses.

\textbf{8.2.3 Study 3: Flicker light-induced retinal vasodilation is unaffected by inhibition of epoxyeicosatrienoic acids and prostaglandins in humans}

Evidence that non-nitric oxide signalling pathways mediate retinal vasodilation during flicker stimulation may suggest novel mechanisms of reduced responses in type 1 diabetes. However, I did not find any evidence to support the hypothesis that EETs or PGs are important mediators of luminance flicker-induced retinal vasodilation. Thus,
dysfunction in their signalling pathways is unlikely to account for reduced flicker responses in people with type 1 diabetes\textsuperscript{3, 12, 13}. Small retinal venule constrictions with fluconazole could indicate that EETs make a small contribution to the regulation of retinal blood flow under physiological conditions. My data indirectly support the hypothesis that nitric oxide is the principal mediator of retinal vasodilation during flicker stimulation. Dysfunctional pathways for nitric oxide therefore appears a more likely mechanism of impaired flicker responses in type 1 diabetes than pathways for EETs or PGs.

8.2.4 Study 4: Retinal neuronal and vascular function in type 1 diabetes during euglycaemic and hyperglycaemic clamp

The focus of Study 4 was on the link between glucose levels (\textbf{Aim 4a}), oxidative stress (\textbf{Aim 4b}) and retinal neurovascular function. My original hypotheses were partially supported. I found good evidence that glucose normalisation with insulin treatment improves luminance flicker-induced retinal venule dilation. These euglycaemic clamps did not significantly affect arteriole responses. Strong within-subject correlations between insulin levels and venule dilation parameters, combined with no significant correlations between glucose levels and venule responses, indicates that this effect was predominantly mediated by insulin infusions rather than glucose-lowering \textit{per se}. My results have similarities to the normalisation of retinal blood flow in people with type 1 diabetes during euglycaemic clamping\textsuperscript{234}. Improved venule dilations may reflect improved retinal microvascular function and blood flow regulation in my type 1 diabetic participants.

In contrast to my hypothesis, hyperglycaemia did not have any significant effect on retinal neurovascular function or systemic arterial elasticity. Other studies have found large, rapid impairments in vascular functional markers with acute hyperglycaemia\textsuperscript{37-39, 231}. I used constant insulin infusions for both euglycaemia and hyperglycaemic clamps to standardise comparisons of glucose levels. In rodents, high insulin levels are protective against acute oxidative stress from hyperglycaemia\textsuperscript{278}. Thus, high insulin levels may have inadvertently protected against the mechanism I was attempting to investigate: the role of hyperglycaemia-induced oxidative stress in reduced luminance flicker-induced retinal vasodilation. I also speculate that a lack of retinal and systemic vascular dysfunction during hyperglycaemia confounded my ability to detect differences between hyperglycaemia with vitamin C and placebo.
Ganglion cell function, measured by the PERG, was generally stable across all glucose conditions. This suggests that ganglion cell dysfunction is predominantly a long-term effect of type 1 diabetes. Curiously, the ratio of the 2F amplitude with 0.8° check sizes to the 2F amplitude with 7° check sizes was significantly increased after hyperglycaemia with vitamin C compared to baseline. The 2F amplitude with 0.8° check sizes is reduced in glaucoma, a disease of retinal ganglion cells, whereas the 2F amplitude with larger check sizes is less affected. Furthermore, the ratio of these amplitudes is a more sensitive predictor of glaucoma than the 0.8° check size 2F amplitude alone. Thus, antioxidants may improve ganglion cell function in type 1 diabetes.

One surprising observation in within-subjects correlation analyses was a positive correlation between plasma glucose levels and arteriole AUC during flicker. I expected these two parameters to be negatively correlated if acute hyperglycaemia impairs vascular functional parameters. Due to the design of Study 4, half of all hyperglycaemic observations were obtained after vitamin C administration. If vitamin C improves luminance flicker-induced retinal arteriole dilation in type 1 diabetes, this could have created a spurious positive correlation between glucose levels and arteriole AUC during flicker. As suspected, when vitamin C data were excluded, this correlation became non-significant. In addition, the exclusion of vitamin C data caused correlations between insulin levels and arteriole dilation parameters to become non-significant.

As yet there are no publications reporting associations between systemic and retinal vascular function. In my study, LAE was positively correlated with retinal arteriole dilation parameters, regardless of the inclusion of vitamin C data. This correlation was despite no significant differences in LAE between glucose conditions. Thus, more elastic retinal blood vessels may be better able to dilate and supply more blood during luminance flicker stimulation. Both LAE and SAE were negatively correlated with systolic blood pressure, consistent with other studies, which suggests that stiffening of the systemic arteries may increase the systolic blood pressure. Systolic blood pressure was not significantly correlated with retinal arteriole or venule dilation parameters. Therefore, it is difficult to attribute correlations between LAE and arteriole dilations to the systolic blood pressure.

PERG amplitudes were not significantly correlated with arteriole and venule dilation parameters in within-subjects analyses. This was not unexpected, given the relative stability of PERG responses across glucose conditions. However, several
correlations emerged between PERG amplitudes and other factors. 7° 2F amplitudes were borderline negatively correlated with insulin levels. This was not the case with 0.8° checks, the size recommended by the ISCEV standard for PERG examinations. Insulin levels were higher towards the end of the protocol and the 7° steady-state response was always recorded last. 7° 2F amplitudes may have declined towards the end of the day due to participant fatigue or ganglion cell habituation. N95 amplitudes were positively correlated with diastolic blood pressures, while 0.8° 2F amplitudes were negatively correlated with systolic blood pressures. However, the correlation with 0.8° 2F amplitudes was no longer significant after the exclusion of data post-vitamin C. The significance of these correlations is uncertain, however it might be possible that retinal ganglion cell function is affected by variations in blood pressure.

Curiously, in baseline tests I often observed an initial venule constriction during the first few seconds of luminance flicker stimulation (Figure 17). I did not observe this response in the venules of non-diabetic people in my earlier studies. This may be due to increased resistance through the capillary network in my diabetic participants. Dilation in the terminal venules from luminance flicker-induced ganglion cell nitric oxide production might promote an initial period of venous blood pooling until the increased flow from the arterial circulation forces this blood back towards the heart. The fact that I favoured superior vessels to avoid upper eyelid interference in my tests would have made gravity-dependent blood pooling away from the vessels more likely.

8.3 Mechanisms of luminance flicker-induced retinal vasodilation

The purpose of my doctoral project was to better understand the mechanisms of luminance flicker-induced retinal vasodilation in humans with and without type 1 diabetes. Based on my results, I formulated a novel hypothesis for the basic mechanisms of this phenomenon. Reduced flicker-induced retinal arteriole dilation with repeat testing and higher ambient lighting suggest that reduced ganglion cell activation leads to smaller increases in retinal blood flow. This is consistent with these responses being mediated by ganglion cells, as previously hypothesised. Furthermore, by showing that EETs and PGs are unlikely to be important signalling molecules in these responses, my research indirectly supports nitric oxide as the main chemical mediator of vasodilation during flicker stimulation. Retinal vasodilation and hyperaemia during luminance flicker stimulation may arise secondary to an NMDA-mediated rise in
ganglion cell intracellular calcium levels, activation of nNOS and release of nitric oxide.

8.3.1 Dysfunction in type 1 diabetes

My results suggest several possible contributors to reduced luminance flicker-induced retinal vasodilation in type 1 diabetes. Insulin infusions improved venule dilations and may have protected against hyperglycaemia-induced vascular dysfunction in my type 1 diabetic participants. Therefore, my data suggest that insulin deficiency in type 1 diabetes may contribute to impaired retinal microvascular function. The mechanism may involve increased iNOS activity and oxidative stress. Improvement in the PERG 2F amplitude 0.8° to 7° ratio after vitamin C suggests a potential benefit of antioxidants on retinal functions. Furthermore, within-subjects correlations between large artery elasticity and flicker-induced retinal arteriole dilation parameters imply that arterial stiffening may lead to impaired neuronal activity-dependent retinal blood flow regulation in type 1 diabetes.

8.4 Strengths and limitations

My doctoral project had several important strengths and limitations. The main strength was that I used interventional studies and designed them around specific hypotheses to increase the knowledge base in areas of ophthalmology. This strategy allowed me to test the role of specific factors in the mechanisms of luminance flicker-induced retinal vasodilation. Conclusions drawn from interventional studies are generally more reliable than those drawn from cross-sectional studies. The latter are useful to find associations between factors at a single point in time but are limited in examining cause-and-effect relationships. Another strength of my research was my focus on the within-subject effects of my interventions. As each participant was essentially the same across all of their characteristics from the start to the conclusion of every study, changes in my study outcomes could be attributed to my interventions, rather than random between-subject factors. The interdependency of observations within each participant is an important statistical feature used in within-subject analyses such as repeated measures ANOVA and paired Student’s t-tests. The design of my studies therefore allowed me to test my hypotheses with good statistical power without the need for hundreds of participants for each separate study. Additional strengths of my research were that I complemented my luminance flicker-induced retinal vasodilation outcomes with clinical and ophthalmic assessments, biochemical investigations and, for
my final study, additional functional assessments of systemic arterial elasticity and retinal ganglion cell function in people with type 1 diabetes. Finally, all of my studies were performed in human subjects, so there is no question about the generalisation of my data to human research, as may be the case for laboratory and animal studies.

My research also had several limitations. It should be recognised that the sample sizes used for my studies were somewhat small, although they were comparable to other human studies of flicker-induced retinal hyperaemia that used within-subject outcomes\textsuperscript{7, 90, 231}. The size of my study samples probably precluded statistically significant findings of reduced venule dilations during flicker stimulation with repeated testing and higher ambient lighting. Regardless, I was able to demonstrate reduced arteriole dilations under these conditions, which implies reduced retinal hyperaemia. The value of including additional observations to improve my power to detect significant reductions in mean venule dilations would be questionable, as this would not affect my conclusions. I appeared to lack sufficient statistical power to find hyperglycaemia-induced reductions in venule dilation with high insulin levels in people with type 1 diabetes. My sample size of 12 participants may not have been sufficient to detect significant changes in this outcome with my study design. Assuming a SD for the change of 1.6%, I would need approximately 82 participants at the two-tailed 5% significance level to have a power of 80% to detect a reduction in venule dilations of 0.5%. At most, I could complete three participants per month under my current design. This would necessitate close to three years to complete 82 participants and is not feasible during the allocated time and available budget for this doctoral work. Furthermore, my primary outcome of arteriole dilations was completely unaffected by hyperglycaemia with high insulin levels. Thus, a new study design is more indicated than a larger sample size to investigate the effects of hyperglycaemia on retinal neurovascular coupling in type 1 diabetes. It is not clear whether my major finding of improved venule dilations during euglycaemic clamp would be generalisable to type 2 diabetes. People with type 2 diabetes are usually insulin resistant and may have other comorbidities, such as hypertension and the drugs used to treat it, that could confound the effects of euglycaemic clamps on retinal neurovascular function\textsuperscript{43}. However, reduced levels of oxidative stress in people with type 2 diabetes during euglycaemic clamps suggest that similar results may be expected\textsuperscript{279}. Similarly, my studies had limited ability to explain results outside the scope of their pre-specified outcomes. For instance, reduced luminance flicker-induced arteriole dilations during repeated testing may have been due to reduced ganglion cell
activation but I did not obtain any electrophysiological data to support this hypothesis. PERG data were obtained for my type 1 diabetes study to address this deficiency. In addition, my small sample sizes made subgroup analyses very difficult to perform effectively. Finally, it should be noted that retinal vasodilation is only a proxy for hyperaemia and retinal blood flow is dependent on both vessel diameters and blood velocities. Thus, I was unable to comment on absolute values of retinal blood flow in my participants. Newer technology may facilitate the direct assessment of retinal blood flow during flicker stimulation.
CHAPTER 9. SIGNIFICANCE AND FUTURE WORK
9.1 Significance of the research

The research described within this doctoral thesis represents three years of original research towards a better understanding of the mechanisms of reduced luminance flicker-induced retinal vasodilation in people with type 1 diabetes. First, I found that this response is reduced with repeated flicker stimulation and higher ambient lighting conditions. Second, I found indirect evidence to support the hypothesis that these responses are primarily mediated by ganglion cell activity and nitric oxide release. Furthermore, my data suggest that impaired retinal neurovascular function may occur with insulin deficiency in type 1 diabetes. My results are also suggestive that antioxidant treatment, specifically vitamin C, may benefit retinal neurovascular function in type 1 diabetes.

This project has important implications for basic and clinical research. Firstly, my project has made an important contribution to the literature on how the retina regulates its own blood flow during periods of increased neuronal stimulation under normal conditions. This will help to guide future basic work in retinal physiology and pharmacology to better understand important fundamental mechanisms. Secondly, I have made an important discovery on the protective role of insulin on retinal neurovascular function in type 1 diabetes. This finding should stimulate interest in the pleiotropic effects of insulin on vascular functions. Thirdly, although most hyperglycaemia with vitamin C data were not statistically significant from euglycaemia in Study 4, my data were suggestive of a small benefit on retinal neurovascular function in people with type 1 diabetes. These observations will be explored in follow-up studies. Finally, my research has led to a better understanding of the mechanisms of luminance flicker-induced retinal vasodilation. This will likely promote the more widespread use of this imaging marker in clinical research.

Dynamic retinal imaging has great potential as a sensitive marker of retinal damage in people with type 1 diabetes. By combining dynamic retinal imaging assessments with traditional ocular assessments, such as clinical ophthalmoscopy and photographic grading, it may be possible to improve risk assessments for the incidence and progression of DR. This could improve the detection and treatment of DR and prevent more people with diabetes from going blind in their most productive years of life.
9.2 Future directions

The results of my doctoral project suggest several new avenues of investigation. If arteriole dilations in my first study were reduced on repeated testing from adaptation to the camera light, electrophysiological tests may help support this hypothesis by quantifying cone and ganglion cell function before and after retinal imaging tests. Cone function can be measured with the full-field ERG. The ISCEV standard advises specific states of dark or light adaptation to standardise comparisons of photoreceptor function, although if changes within each participant are of interest this might not be essential. Ganglion cell function can be measured with the PERG, as in my type 1 diabetes study, but this is done with an undilated pupil. The PhNR of the full-field ERG is an alternative marker of ganglion cell function that can be conducted with a dilated pupil. Given that dynamic retinal imaging is generally performed with a dilated pupil, the PhNR may be more useful for correlating ganglion cell function with flicker-induced vasodilations. I would expect cone responses and ganglion cell responses to be attenuated after dynamic retinal imaging tests, indicating adaptation and reduced sensitivity to light stimuli. A similar strategy could be used to investigate whether ganglion cell activity is attenuated when flicker stimulation is delivered under higher ambient lighting. The PERG steady-state response, which uses a rapidly alternating checkerboard stimulus, may be most useful for comparing ganglion cell activation under different levels of ambient lighting.

I did not find any evidence that EETs or PGs are important mediators of flicker light-induced retinal vasodilation, as suggested by ex vivo rodent studies. Additional in vivo animal studies are therefore required to confirm the importance, or lack thereof, of these arachidonic acid metabolites in flicker-induced retinal vasodilation. My data support the hypothesis that luminance flicker-induced retinal vasodilation is mediated by nitric oxide. The enzymes and cells responsible for retinal nitric oxide release during flicker stimulation remain unclear, so additional laboratory work is needed to separate the contributions of different NOS enzymes and cells to these responses. Ganglion cell nitric oxide production is likely to be a significant mediator of these responses. Thus, experiments should specifically investigate ganglion cell nitric oxide production during flicker stimulation and the potential importance of NMDA receptors as the regulators of neuronal intracellular calcium.

Unfortunately my type 1 diabetes study was not able to demonstrate that hyperglycaemia impairs retinal neurovascular function. I attribute this to the stabilising
effects of high insulin levels on iNOS activity and oxidative stress. Hyperglycaemic clamp studies with lower or normal insulin levels are required to further elucidate this relationship. If the primary aim were to study the effects of hyperglycaemia on retinal neurovascular function, it would be reasonable to induce acute hyperglycaemia in the absence of insulin infusions in people with type 1 diabetes. This would simplify procedures, reduce the time needed for each study and be more convenient for participants.

Antioxidants such as vitamin C may improve retinal neurovascular function in type 1 diabetes. Other hyperglycaemic clamp studies have found protection of systemic arterial stiffness\textsuperscript{37} and endothelial function\textsuperscript{40} with vitamin C. However, my study was underpowered to detect significant differences between the vitamin C and placebo arms in hyperglycaemia. I speculate that this was largely due to the protective effects of pharmacologic doses of insulin. Studies to assess retinal functional parameters with and without vitamin C are merited. The effects of vitamin C should also be investigated in people with and without type 1 diabetes to see if effects are unique to type 1 diabetes.

Advances in imaging technology may improve retinal functional assessments. Doppler-OCT devices now allow the direct quantification of blood flow in retinal blood vessels. When combined with scanning laser ophthalmoscopy, it is possible to directly quantify central retinal artery and vein blood flow perpendicular to the imaging plane. However, simultaneous Doppler-OCT imaging and flicker stimulation is still in development. Adaptive optics imaging compensates for subtle aberrations in the refractive media of an individual eye. With this technology, it is possible to obtain detailed images of microscopic retinal structures down to the resolution of an individual photoreceptor. Adaptive optics imaging could be used to greatly improve the accuracy of retinal vascular studies. Functional magnetic resonance imaging (fMRI) could also be used in humans to study the activation of parasol and midget retinal ganglion cells during different forms of flicker stimulation. fMRI measures brain activity by detecting changes in blood flow. This technology could be used to study activity in the LGN: magnocellular activity would imply parasol activity in the retina, while parvocellular activity would imply midget activity in the retina.

Several cross-sectional studies have observed reduced arteriole and venule dilations during luminance flicker stimulation in people with diabetes compared with non-diabetic control subjects\textsuperscript{13, 17-19}. However, no study has yet evaluated the potential predictive value of these functional markers on the incidence and progression of DR.
Longitudinal clinical studies are therefore needed to evaluate the benefit of retinal neuronal and vascular functional parameters versus clinical or photographic grading for the clinical management of DR.
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Noonan, Jonathan Edward

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